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February 8, 2013

TSCA Confidential Business Information Center (7407M)
EPA East - Room 6428 Attn: Section 8(e)
U.S. Environmental Protection Agency
1200 Pennsylvania Avenue, N.W.
Washington, DC 20460-0001

Re: Submission Pursuant to Section 8(e) of the Toxic Substances Control Act ("TSCA"): Fresh Water Algal Growth Inhibition Study for Amines, bis (C11-14-branched and linear alkyl), tungstates

Dear Sir or Madam:

On January 9, 2013, [redacted] ("the Company") received a final draft of an algal growth inhibition study ("Final Study") for one of the substances used in [redacted] ("The Product").[1] As described in the original pre-manufacture notice, the specific substance tested ("Tested Substance") is identified as "Amines, bis (C11-14-branched and linear alkyl), tungstates"[2] and its CAS registry number is 1159919-46-6. The testing laboratory, NOTOX B.V. ("NOTOX"), assessed the Tested Substance for Fresh Water Algal Growth Inhibition using Pseudokirchneriella subcapitata. The study procedures described in this report were based on the OECD guideline No. 201, 2006; Annex 5 corrected 28 July 2011. In addition, the procedures were designed to meet the test methods of the Commission Regulation (EC) No 440/2008, Part C.3, 2008; Amended by EC No. 761/2009, the ISO International Standard 8692, 2004 and the OECD series on testing and assessment number 23, 2000.

The findings of the Final Study are consistent with the findings reported in the Draft Study, previously submitted to EPA on June 25, 2012. The Final Study established the following toxicity parameters for the Tested Substance: The EC50 for growth rate reduction (ERC50: 0-

[1] Portions of this letter claimed as confidential are bracketed and highlighted in **bold.**

[2] The Study itself, conducted in Europe, includes references to an alternative descriptive formula prepared for a confidential submission to the European Chemicals Agency under REACH. *See* Study at 8 and 53. Because this alternative descriptive formula was not included in the pre-manufacture notice ("PMN") or on the TSCA inventory, and because the alternative nomenclature would provide competitors with information relevant to the manufacturing process for the substance itself, the Company is claiming it as confidential business information ("CBI"), and has annotated the public version of the study to reference the nonconfidential US nomenclature.

Company Sanitized

72h) was estimated to correspond to 0.88 µg/l. The EC50 for yield inhibition (EYC50: 0-72h) was 0.36 µg/l with a 95% confidence interval ranging from 0.17 to 0.76 µg/l. The NOEC based on TWA concentrations for both growth rate reduction and yield inhibition was 0.15 µg/l, derived from a nominal test concentration of 1.8 mg/L.

Please note that the Company has not made a determination as to whether a substantial risk of injury to health or the environment is actually presented by these findings. Recognizing, however, that EPA could interpret such information as constituting a substantial risk when considered with other studies submitted to the Agency, the Company is submitting the study EPA under TSCA §8(e) out of an abundance of caution.

Enclosed are confidential and redacted public versions of the Final Study and Protocol, this cover letter, and a detailed justification for confidential treatment of the Company's identifying information, the trade name for the Product containing the Tested Substance, and the alternative descriptive formula.

If you have any questions or need more information, please contact me at 203-295-2143 Ext 264.

Sincerely yours,

[redacted]

Enclosures

cc: [redacted]

Attachment 1: Substantiation for Confidentiality Claims

Substantiation Questions

1. **Is your company asserting this confidential business information (CBI) claim on its own behalf? If the answer is no, please provide company name, address and telephone number of entity asserting claim.**

   Company asserts this CBI claim on its own behalf.

2. **For what period do you assert your claim(s) of confidentiality? If the claim is to extend until a certain event or point in time, please indicate that event or time period. Explain why such information should remain confidential until such point.**

   The Company asserts an indefinite claim of confidentiality with respect to three categories of information: a) The Company's name and address; b) the Trade Name and identity of a proprietary product containing the Tested Substance, and c) an alternative descriptive formula for the Tested Substance prepared by NOTOX for a confidential submission to the European Chemicals Agency. Each is discussed in turn.

   a. **The Company's name, address, and other identifying information.**

      The cover letter and pages 7, 50, and 51 of the Study reference the Company's name and address. The Company claims this information as CBI for an indefinite period.

      Disclosure of Company information and the Product Trade Name would disclose confidential business information relating to the Company's extensive research, development, and commercialization efforts to evaluate, identify, and select specific substances with exceptional performance characteristics in competitive markets. Disclosure would also provide competitors with sensitive and confidential information on specific details of the proprietary ingredients used in specific Company products. Disclosure of the name of the Company submitting this test would also disclose confidential business information regarding business relationships the Company has established with specific third-party testing laboratories.

   b. **The Trade Name and identity of a Proprietary Product.**

      The cover letter and each page of the Study reference the Trade Name and identity of a Product containing the Tested Substance. The Company claims this information to be CBI for an indefinite period.

      As with the Company information, disclosure of the Product Trade Name would release confidential business information relating to the Company's extensive research, development, and commercialization efforts to evaluate, identify, and select specific substances with exceptional performance characteristics in competitive markets. Disclosure of Company and Product Trade Names would

also provide competitors with sensitive and confidential information on specific details of the proprietary ingredients used in specific Company products. Finally, disclosing the Product Name would, in turn, disclose the identity of the Company that commissioned this test, thus disclosing CBI regarding business relationships the Company has established with specific third-party testing laboratories.

c. **Alternative Descriptive Formula.**

Page 8 and 53 of the Study reference an alternative descriptive formula for the Tested Substance prepared by NOTOX as part of a confidential submission to the European Chemicals Agency under REACH. The alternative descriptive formula refers to the same Tested Substance identified in the Company's PMN and the TSCA Inventory as "Amines, bis(C11-14-branched and linear alkyl), tungstates," CAS No. 1159919-46-6. Unlike the descriptive formula contained in the PMN and on the TSCA Inventory, however, the confidential alternative descriptive formula would provide competitors with more detailed information that could reveal elements of the manufacturing process for the substance itself. As such, the Company is claiming the alternative descriptive formula to be confidential business information and has annotated the public version of the study to include reference to the nonconfidential US nomenclature.

3. **Has the information that you are claiming as CBI been disclosed to any other Governmental Agency, or to this Agency at any other time?**

The trade name was disclosed on the PMN, but was marked as CBI. The trade name and alternative descriptive formula were disclosed in a submission to the Environmental Chemicals Agency pursuant to Article 26 of REACH. This submission, however, was submitted as confidential and is not available to the public.

4. **Briefly describe any physical or procedural restrictions within your company relating to the use and storage of the information you are claiming CBI.**

CBI is kept secure in locked file cabinets and its distribution is restricted to company personnel on an as need to know basis. All computer networks containing information are secured and protected by firewalls.

5. **If anyone outside your company has access to any of the information claimed CBI, are they restricted by confidentiality agreement(s)? If so, explain the content of the agreement(s).**

While the Tested Substance is listed on the Toxic Substances Inventory, its use in the Product is CBI. Such information is shared with vendors on a need to know basis, and only under stipulations of confidentiality preventing the distribution of such information.

6. **Does the information claimed as CBI appear or is it referred to in any of the following:**

   a. **Advertising or promotional material for the chemical substance or the resulting end product;**

      No. These materials would not link the Tested Substance to the Company or a specific Company Product by CAS Number or substance composition.

   b. **Material safety data sheets or other similar materials (such as technical data sheets) for the substance or resulting end product (include copies of this information as it appears when accompanying the substance and/or product at the time of transfer or sale);**

      No. These materials would not link the Tested Substance to the Company or a specific Company Product by CAS number or substance composition.

   c. **Professional or trade publications; or**

      No. These materials would not link the Tested Substance to the Company or a specific Company Product by CAS Number or substance composition.

   d. **Any other media or publications available to the public or to your competitors.**

      No. These materials would not link the Tested Substance to the Company or a specific Company Product by CAS Number or substance composition.

7. **Has EPA, another federal agency, or court made any confidentiality determination regarding information associated with this substance? If so, provide copies of such determinations.**

   No.

8. **Describe the substantial harmful effects that would result to your competitive position if the CBI is made available to the public.**

   As noted above, the Company is not seeking to limit the public availability of the health and safety data in the study or the CAS number and name of the specific substance tested. Rather, the CBI claims extend to the identifying information for the Company itself and the Proprietary Product(s) in which the Tested Substance is used. The Company expends considerable resources on research and development to identify which substances provide the highest level of performance and value for customers, and this information would have significant value to competitors seeking to compete in similar markets. While the CAS Number and structural identity of the Tested Substance is publicly available, releasing information on its use in specific Company products would undermine the Company's competitive advantage by implicitly disclosing proprietary information on the value and utility of the substance for specific market uses. The

alternative descriptive formula, not required for the domestic registration, would provide competitors with additional information governing the manufacturing process for the substance.

9. **Has the substance been patented in the U.S. or elsewhere? Is a patent for the substance currently pending?**

Composition of matter patent. Patent filed and granted in the US. Patents filed and granted in India, Japan, China and Germany. These documents do not disclose the information claimed as CBI in this filing.

10. **Is this substance/product commercially available and if so, for how long has it been available on the commercial market?**

Yes. The Tested Substance has been commercially available since May 2009.

If on the commercial market, are your competitors aware that the substance is commercially available in the U.S.?

The MSDS states that the product contains "amines bis(C11-C14 alkyl) tungstates," but does not link the product to a specific CAS No. or disclose the specific product composition beyond a range.

a. **If not already commercially available, describe what stage of research and development (R&D) the substance is in, and estimate how soon a market will be established.**

[redacted]

b. **What is the substance used for and what type of product(s) does it appear in?**

[redacted]

11. **Describe whether a competitor could employ reverse engineering to identically recreate the substance.**

The Company does not oppose disclosing the identity of the substance itself, and it has not sought to claim the CAS number or the name as submitted in the PMN and as listed on the TSCA Inventory. Rather, the Company is concerned that disclosing the identity of the Manufacturer, the Trade Name of the Product in which it is used, and the alternative descriptive formula would allow a competitor to deduce confidential properties and commercial values of the Tested Substance. Moreover, the alternative descriptive formula would assist a competitor in determining the manufacturing process for the Tested Substance.

12. Do you assert that disclosure of this information you are claiming CBI would reveal:

a. Confidential processes used in manufacturing the substance;

Disclosure of the alternative descriptive formula would compromise confidential information regarding the manufacturing process for the substance.

b. If a mixture, the actual portions of the substance in the mixture; or

As stated on MSDS petroleum process oil, <3.0%, DMSO extractable material 64742-52-5 40 – 70% amines bis(C11-C14 alkyl) tungstates 30 – 60%.

c. Information unrelated to the effects of the substance on human health or the environment?

The Company does not oppose disclosing the identity of the substance itself, and it has not sought to claim the CAS number or substance name, as filed in the PMN, as CBI. Nor does the Company seek to restrict public access to the Study's findings on the potential effects of the Tested Substance on human health or the environment. Rather, the Company is concerned that disclosing the identity of the Manufacturer, the Trade Name of a product in which the Tested Substance is used, and the alternative descriptive formula would reveal sensitive market and economic information on the value of specific substances to specific market uses, its presence in specific proprietary Company products, and information regarding the manufacturing process.

13. Provide the Chemical Abstract Service Registry Number for the product, if known.

1159919-46-6.

14. Is the substance or any information claimed CBI the subject of FIFRA regulation or reporting? If so, explain.

No.

FINAL REPORT

Study Title

FRESH WATER ALGAL GROWTH INHIBITION TEST WITH [redacted] (OIL FREE)

Author

Ing. M.H.J. Migchielsen

Test Facility

NOTOX B.V.
Hambakenwetering 7
5231 DD 's-Hertogenbosch
The Netherlands

Laboratory Project Identification

NOTOX Project 498471
NOTOX Substance 203662/A

1. CONTENTS

TABLES

FIGURES

APPENDICES

2. STATEMENT OF GLP COMPLIANCE

NOTOX B.V., 's-Hertogenbosch, The Netherlands

The study described in this report has been correctly reported and was conducted in compliance with:

The Organization for Economic Co-operation and Development (OECD) Principles of Good Laboratory Practice (GLP) (as revised in 1997) ENV/MC/CHEM (98) 17.

The sponsor is responsible for Good Laboratory Practice (GLP) compliance for all test substance information unless determined by NOTOX.

NOTOX B.V.

Ing. M.H.J. Migchielsen
Study Director

Date: 23 August 2012

Ing. E.J. van de Waart, M.Sc.
Head of In Vitro & Environmental Toxicology

i.a. i.A.T.H. Heerts
Section Head

Date: 22 August 2012

3. QUALITY ASSURANCE STATEMENT

NOTOX B.V., 's-Hertogenbosch, The Netherlands

This report was inspected by the NOTOX Quality Assurance Unit to confirm that the methods and results accurately and completely reflect the raw data.

During the on-site process inspections, procedures applicable to this type of study were inspected.

The dates of Quality Assurance inspections are given below.

Type of Inspections	Phase/Process	Start Inspection date	End Inspection date	Reporting date
Study	Protocol	20-Jan-12	20-Jan-12	20-Jan-12
	Report	03-May-12	03-May-12	03-May-12
	Protocol Amendment 1	17-Aug-12	17-Aug-12	17-Aug-12
Process	**Environmental Toxicology** Test Substance Handling Exposure Observations/Measurements	30-Jan-12	03-Feb-12	03-Feb-12
	Analytical and physical chemistry Test Substance Handling Observations/Measurements	06-Feb-12	13-Feb-12	16-Feb-12

NOTOX B.V.

C.J. Mitchell B.Sc.
Head of Quality Assurance

Date: 22-Aug-2012.....

4. SUMMARY

Pseudokirchneriella subcapitata, Fresh Water Algal Growth Inhibition Test with [redacted] (oil free).

The study procedures described in this report were based on the OECD guideline No. 201, 2006; Annex 5 corrected 28 July 2011. In addition, the procedures were designed to meet the test methods of the Commission Regulation (EC) No 440/2008, Part C.3, 2008; Amended by EC No. 761/2009, the ISO International Standard 8692, 2004 and the OECD series on testing and assessment number 23, 2000.

The batch of [redacted] (oil free) tested was a UVCB substance. The material was not completely soluble in the test medium at the initial loading rates prepared (indicated as "insoluble in cold water" on MSDS).

Preparation of test solutions started with individually prepared loading rates. Exact amounts of the viscous liquid were weighed and placed on cover slips. The cover slips were then transferred into measuring flasks that contained pre-heated (35-39°C) test medium. Subsequently, a three-day magnetic stirring period was applied to ensure reaching maximum dissolution in test medium at the various loading rates. The resulting dispersions were left to settle for 1-2 hours were after the Water Accommodated Fractions (WAFs) were collected and used for testing. The final test solutions were all clear and colourless.

An initial main test was performed based on the results of a preceding combined limit/range-finding test. Six replicates of exponentially growing algal cultures were exposed to a control and three replicates per test group were exposed to WAFs prepared at loading rates of 1.0, 1.8, 3.2, 5.6 and 10 mg/l. The total test period was 72 hours and the initial algal cell density was 10^4 cells/ml. Samples for analyses of actual exposure concentrations were taken at the start, after 24 and 72 hours of exposure. Due to highly variable growth results obtained in the first main test, it was decided to perform an additional main test with the same test set-up as the first test.

Analyses of the samples taken at the start of the two main tests showed measured concentrations that varied between 0.35 and 275 µg/l for the WAFs prepared at 1.0, 1.8, 3.2, 5.6 and 10 mg/l. There was no relationship between the measured concentrations and the initial loading rates prepared. The highly variable concentrations measured were expected to be due to the very poor solubility of the UVCB test substance and its stickiness (viscous liquid). Measured concentrations generally decreased during the test period but also some fluctuations were observed in measurements after 24 and 72 hours indicating that test solutions were likely inhomogeneous. At the end of the test high variations in algal cell density were also observed in individual replicates of some of the WAFs. This is likely related to the variability in the concentrations of the test substance. Time Weighted Average (TWA) exposure concentrations in the first main test corresponded to 1.9, 0.38, 2.9, 0.22 and 8.5 µg/l for the WAFs prepared at 1.0, 1.8, 3.2, 5.6 and 10 mg/l. In the second test TWA concentrations corresponded to 4.4, 0.15, 4.5, 0.98 and 1.9 µg/l for the WAFs prepared at 1.0, 1.8, 3.2, 5.6 and 10 mg/l.

The study met the acceptability criteria prescribed by the protocol and was considered valid.

Under the conditions of the present study with *Pseudokirchneriella subcapitata* exposed to various [redacted] (oil free) concentrations, the following toxicity parameters were determined:

The EC_{50} for growth rate reduction (E_RC_{50}: 0-72h) was estimated to correspond to 0.88 µg/l.

The EC_{50} for yield inhibition (E_YC_{50}: 0-72h) was 0.36 µg/l with a 95% confidence interval ranging from 0.17 to 0.76 µg/l.

The NOEC based on TWA concentrations for both growth rate reduction and yield inhibition was 0.15 µg/l. This NOEC is derived from a loading rate (nominal test concentration) of 1.8 mg/l.

Note that results were based on two main studies and that a worst-case approach was followed to determine the EC and NOEC values.

5. INTRODUCTION

5.1. Preface

Sponsor	[redacted]
Study Monitor	Mr. R. Balcomb Director, Toxicology and Environmental Assessments Intertek Regulatory Services 1035 17th Street No.4 SANTA MONICA, CA 90403 USA
Test Facility	NOTOX B.V. Hambakenwetering 7 5231 DD 's-Hertogenbosch The Netherlands
Study Director	Ing. M.H.J. Migchielsen
Principal Scientist	E. Baltussen, PhD
Study Plan	Start : 06 February 2012 Completion : 29 March 2012

5.2. Aim of the study

The purpose of the study is to evaluate the test substance for its ability to generate toxic effects in *Pseudokirchneriella subcapitata* during an exposure period of at least 48 and at most 96 hours and, if possible, to determine the EC_{50} for both reduction of growth rate and inhibition of yield.

5.3. Guidelines

The study procedures described in this report were based on the Organization for Economic Co-operation and Development (OECD), OECD guidelines for Testing of Chemicals, guideline No. 201: "Freshwater Alga and Cyanobacteria, Growth Inhibition Test", Adopted March 23, 2006; Annex 5 corrected 28 July 2011.

In addition, the procedures were designed to meet the test methods prescribed by the following guidelines and guidance document:

- Commission regulation (EC) No. 440/2008 of 30 May 2008, Part C: Methods for the determination of ecotoxicity, Publication No. L142, C3: "Algal Inhibition Test"; Amended by EC No. 761/2009 of 23 July 2009, Publication No. L220.
- ISO International Standard 8692: "Water quality - Freshwater algal growth inhibition test with unicellular green algae", Second edition, 01 October 2004.
- Guidance document on aquatic toxicity testing of difficult substances and mixtures, OECD series on testing and assessment number 23, December 14, 2000.

5.4. Storage and retention of records and materials

Records and materials pertaining to the study including protocol, raw data, specimens (except specimens requiring refrigeration or freezing) and the final report are retained in the NOTOX archives for a period of at least 2 years after finalization of the report. After this period, the sponsor will be contacted to determine how the records and materials should be handled. NOTOX will retain information concerning decisions made.

Those specimens requiring refrigeration or freezing will be retained by NOTOX for as long as the quality of the specimens permits evaluation but no longer than three months after finalization of the report.

NOTOX will retain a test substance sample until the expiry date, but no longer than 10 years after finalization of the report. After this period the sample will be destroyed.

5.5. Definitions

Cell density is the number of cells per millilitre.

Growth rate is the increase in cell density per unit time. It is derived from the slope of the growth curve in a logarithmic plot. Following from the mathematical nature of exponential growth, the measure of the specific growth rate is preferable over biomass or yield. The E_RC_{50} is the concentration of test substance that results in a 50% reduction in growth rate relative to the control.

Yield is defined as the biomass at the end of the exposure period minus the biomass at the start of the exposure period. The E_yC_{50} is the concentration of test substance that results in a 50% inhibition of yield relative to the control.

No Observed Effect Concentration (NOEC) is the highest concentration tested at which the measured parameter(s) show(s) no significant effect on algal growth relative to control values.

6. MATERIALS AND METHODS

6.1. Test Substance

6.1.1. Test substance information

Cite publicly per PMN and TSCA Inventory list nomenclature as: Amines, bis (C11-14-branched and linear alkyl) tungstates.

Identification	[redacted] (oil free)
Molecular formula	[redacted]
CAS Number	1159919-46-6
Description	Clear yellow viscous liquid (determined at NOTOX)
Batch	PB-39-131
Purity	UVCB
Test substance storage	At room temperature in the dark
Stability under storage conditions	Stable
Expiry date	01 December 2012 (allocated by NOTOX, 1 year after receipt of the test substance)

6.1.1. Study specific test substance information

Volatile	Not indicated
Stability at higher temperatures	Not indicated
Stability in water	Not indicated
Solubility in water	Insoluble

6.1.2. Reference substance

This report includes the results of the most recent reference test with potassium dichromate (APPENDIX 4).

6.2. Test System

Species	*Pseudokirchneriella subcapitata*, strain: NIVA CHL 1
Source	In-house laboratory culture.

Reason for selection

This system is an unicellular algal species sensitive to toxic substances in the aquatic ecosystem and has been selected as an internationally accepted species.

6.3. Fresh water algae culture

Stock culture

Algae stock cultures were started by inoculating growth medium with algal cells from a pure culture on agar. The suspensions were continuously aerated and exposed to light in a climate room at a temperature of 21-24°C.

Light intensity

60 to 120 µE/m^2/s when measured in the photosynthetically effective wavelength range of 400 to 700 nm.

Stock culture medium

M1; according to the NPR 6505 ("Nederlandse Praktijk Richtlijn no. 6505") formulated using Milli-RO water (tap-water purified by reverse osmosis; Millipore Corp., Bedford, Mass., USA) with the following composition:

$NaNO_3$	500	mg/l
K_2HPO_4 $3H_2O$	52	mg/l
$MgSO_4$ $7H_2O$	75	mg/l
$Na_2CO_3.10H_2O$	54	mg/l
$C_6H_8O_7.H_2O$	6	mg/l
NH_4NO_3	330	mg/l
$CaCl_2.2H_2O$	35	mg/l
$C_6H_5FeO_7.xH_2O$	6	mg/l
H_3BO_3	2.9	mg/l
$MnCl_2.4H_2O$	1 81	mg/l
$ZnCl_2$	0 11	mg/l
$CuSO_4$ $5H_2O$	0.08	mg/l
$(NH_4)_6Mo_7O_{24}.4H_2O$	0.018	mg/l

Pre-culture

3 days before the start of the test, cells from the algal stock culture were inoculated in culture medium at a cell density of 1 x 10^4 cells/ml. The pre-culture was maintained under the same conditions as used in the test. The cell density was measured immediately before use.

Pre-culture medium

M2; according to the OECD 201 Guideline, formulated using Milli-Q water (tap water purified by reverse osmosis (Milli-RO) and subsequently passed over activated carbon and ion-exchange cartridges: Milli-Q water; Millipore Corp., Bedford, Mass., USA) preventing precipitation and with the following composition:

NH_4Cl	15	mg/l
$MgCl_2$ $6H_2O$	12	mg/l
$CaCl_2$ $2H_2O$	18	mg/l
$MgSO_4.7H_2O$	15	mg/l
KH_2PO_4	1.6	mg/l
$FeCl_3.6H_2O$	64	µg/l
$Na_2EDTA.2H_2O$	100	µg/l
H_3BO_3	185	µg/l
$MnCl_2.4H_2O$	415	µg/l
$ZnCl_2$	3	µg/l
$CoCl_2.6H_2O$	1 5	µg/l
$CuCl_2.2H_2O$	0 01	µg/l
$Na_2MoO_4.2H_2O$	7	µg/l
$NaHCO_3$	50	mg/l
Hardness (Ca+Mg)	0.24	mmol/l (24 mg $CaCO_3$/l)
pH	8.1 ± 0.2	

6.4. Preparation of test solutions

The standard test procedures required generation of test solutions, which contained completely dissolved test substance concentrations or stable and homogeneous mixtures or dispersions. The testing of concentrations that would disturb the test system was prevented as much as possible (e.g. film of the test substance on the water surface).

The batch of [redacted] (oil free) tested was a UVCB substance. The material was not completely soluble in the test medium at the initial loading rates prepared (indicated as "insoluble in cold water" on MSDS).

Preparation of test solutions started with individually prepared loading rates. Exact amounts of the viscous liquid were weighed and placed on cover slips. The cover slips were then transferred into measuring flasks that contained pre-heated (35-39°C) test medium. Subsequently, a three-day magnetic stirring period was applied to ensure reaching maximum dissolution in test medium at the various loading rates. The resulting dispersions were left to settle for 1-2 hours were after the Water Accommodated Fractions (WAFs) were collected and used for testing. The final test solutions were all clear and colourless.

After preparation, volumes of 50 ml were added to each replicate of the respective test concentration. Subsequently, 1 ml of an algal suspension was added to each replicate providing a cell density of 10^4 cells/ml.

6.5. Combined limit/range-finding test

The project started with a combined limit/range-finding test. Six replicates of exponentially growing algae were exposed to a control and a WAF prepared at a loading rate of 100 mg/l. Test procedure and conditions were similar to those applied in the final test with the following exceptions:

- Three replicates per concentration were exposed to WAFs prepared at loading rates of 1.0 and 10 mg/l.
- One extra test vessel per concentration without algae was used as background for the determination of the algal cell density at each time interval.
- pH was only measured in the control and the highest test concentration.
- At the end of the test algae were not observed to verify a normal and healthy appearance.

6.6. Final tests

6.6.1. Test concentrations

[redacted] (oil free)	WAFs prepared at loading rates of 0.46, 1.0, 2.2, 4.6 and 10 mg/l.
Controls	Test medium without test substance or other additives.
Replicates	3 replicates of each test concentration, 6 replicates of the control, 1 extra replicate of each concentration for sampling after 24 hours, 1 replicate of the highest concentration without algae.

6.6.2. Test procedures and conditions

Test duration	72 hours
Test type	Static
Test vessels	100 ml, all-glass, containing 50 ml of test solution
Medium	M2

Cell density	An initial cell density of 1 x 10^4 cells/ml.
Illumination	Continuously using TLD-lamps of the type 'Cool-white' of 30 Watt, with a light intensity within the range of 64 to 72 $\mu E.m^{-2}.s^{-1}$.
Incubation	Capped vessels were distributed at random in the incubator and as such were daily repositioned. During incubation the algal cells were kept in suspension by continuous shaking.

6.6.3. Sampling for analysis of test concentrations

During the two final tests singular samples for possible analysis were taken from all test concentrations and the control according to the schedule below. The method of analysis is described in the appended Analytical Report (APPENDIX 5).

Frequency	at t=0 h, t=24 h and t=72 h
Volume	1 ml
Storage	Samples were stored in a freezer until analysis.

At the end of the exposure period, the replicates with algae were generally pooled at each concentration before sampling, except for incidental replicates that showed large differences in algal density.

Compliance with the Quality criteria regarding maintenance of actual concentrations was demonstrated by running a test vessel at the highest substance concentration but without algae and samples for analysis were taken at the start, after 24 hours of exposure and at the end of the test period.

Additionally, singular reserve samples of 1 ml were taken from all test solutions for possible analysis. If not already used, these samples were stored in a freezer for a maximum of three months after delivery of the draft report, pending on the decision of the sponsor for additional analysis.

6.6.4. Measurements

pH	At the beginning and at the end of the test. The pH of the solutions should preferably not deviate by more than 1.5 units during the test.
Temperature of medium	Continuously in a temperature control vessel.
Appearance of the cells	At the end of the final tests microscopic observations were performed to observe for any abnormal appearance of the algae.

6.6.5. Recording of cell densities

At the beginning of both final tests, cells were counted using a microscope and a counting chamber. In final test 1, cell densities were determined by spectrophotometric measurement of samples at 720 nm using a spectrophotometer with immersion probe (pathlength =20 mm). Algal medium was used as blank. The test solutions in final test 2 contained undissolved particles that disturbed spectrophotometric measurement. Therefore, algal density was determined by use of a microscope and a counting chamber throughout the 2nd final test.

6.7. Electronic data capture

Observations/measurements in the study were recorded electronically using the following programme(s):
- Shimadzu Spectrophotometer UV-1800 including UVProbe 2.33 software (Shimadzu, Kyoto, Japan): Algal cell density.
- REES Centron Environmental Monitoring system version SQL 2.0 (REES Scientific, Trenton, NJ, USA): Temperature.

6.8. Interpretation

6.8.1. Data handling

Calibration curve
Quantification of cell densities in the first final test was based on a calibration curve. Cell density was plotted versus extinction using spectrophotometric measurements of a minimum of six dilutions of an algal suspension with different cell densities. The calibration curve was composed using linear regression. The software automatically calculates the cell densities based on this curve for the spectrophotometric measurements at the various points in time during the test period.

Comparison of average growth rates
The average specific growth rate for a specific period is calculated as the logarithmic increase in the biomass from the equation for each single vessel of controls and treatments:

$$\mu_{i-j} = \frac{\ln X_j - \ln X_i}{t_j - t_i} (\text{day}^{-1})$$

Where: μ_{i-j} = the average specific growth rate from time i to j
X_i = the biomass at time i
X_j = the biomass at time j

The average growth rate at each test substance concentration is then compared with the control value and the percentage reduction in growth rate is calculated:

$$\%I_r = \frac{\mu_C - \mu_T}{\mu_C} \times 100$$

Where: $\%I_r$ = percent inhibition in average specific growth rate
μ_C = mean value for average specific growth rate in the control group
μ_T = average specific growth rate for the treatment replicate

Yield
The percent inhibition in yield is calculated for each treatment replicate as follows:

$$\%I_y = \frac{Y_C - Y_T}{Y_C} \times 100$$

Where: $\%I_y$ = percent inhibition of yield
Y_C = mean value for yield in the control group
Y_T = value for yield for the treatment replicate

Determination of the average exposure concentrations

The average exposure concentrations were calculated as:

$\frac{24 \times \sqrt{C_{t=0} \times C_{t=24}} + 48 \times \sqrt{C_{t=24} \times C_{t=72}}}{72}$, being the Time Weight Average (TWA) of the concentrations of [redacted] (oil free) measured in the samples taken at the start ($C_{t=0}$), after 24 hours ($C_{t=24}$) and the end of the test ($C_{t=72}$).

Determination of the NOEC and calculation of the EC_{50}
For determination of the NOEC and the EC_{50} the approaches recommended in the OECD guideline 201 were used. An effect was considered to be significant if statistical analysis of the data obtained for the test concentrations compared with those obtained in the negative control revealed significant reduction of growth rate or inhibition of yield (ANOVA, Bonferroni t-test, TOXSTAT Release 3.5, 1996, D.D. Gulley, A.M. Boelter, H.L. Bergman). Additionally, the EC_{10} was determined to meet the recommendations as put down in "A Review of Statistical Data Analysis and Experimental Design in OECD Aquatic Toxicology Test Guidelines" by S. Pack, August 1993.
Calculation of the EC_{50} and EC_{10} values was based on log-linear regression analysis of the percentages of growth rate reduction and the percentages of yield inhibition versus the logarithms of the corresponding average exposure concentrations of the test substance.

6.8.2. Acceptability of the test

1. In the controls, cell density increased by an average factor of > 16 within 2 days.
2. The mean coefficient of variation for section-by-section specific growth rates in the control cultures did not exceed 35%.
3. The coefficient of variation of average specific growth rates during the whole test period in replicate control cultures did not exceed 7%.

6.9. List of deviations

6.9.1. List of protocol deviations

There were no deviations from the protocol.

6.9.2. List of standard operating procedures deviations

Any deviations from standard operating procedures were evaluated and filed in the study file. There were no deviations from standard operating procedures that affected the integrity of the study.

7. RESULTS

7.1. Combined limit/range-finding test

7.1.1. Mean cell densities, reduction of growth rate and inhibition of yield

The mean cell densities measured during the combined limit/range-finding test are presented in Table 1. Table 2 presents the percentages growth rate reduction and yield inhibition per concentration. Algal growth was completely inhibited in the WAFs prepared at 10 and 100 mg/l, while no significant effects on growth were observed in the WAF prepared at 1.0 mg/l.

Analyses of the samples taken from the WAFs prepared at 1.0 and 10 mg/l at the start of the test showed measured concentrations of 0.7 and 57 µg/l, respectively. The concentration in the WAF prepared at 1.0 mg/l remained stable during the exposure period at 0.7 µg/l, while the measured concentration in the WAF prepared at 10 mg/l decreased to 6.2 µg/l (see also Table 2 of the appended Analytical report).

The expected EC_{50} for growth rate reduction was between concentrations present in WAFs prepared at 1.0 and 10 mg/l. Based on expected actual concentrations this corresponded to a range between 0.7 and 57 µg/l. All test conditions were maintained within the limits prescribed by the protocol.

Table 1 Mean cell densities (x10^4 cells/ml) during the combined limit/range-finding test

Loading rate [redacted] (oil free) WAF (mg/l)	Exposure time (hours)			
	0	24	48	72
Control	1 0	13.4	67.8	257.6
1.0	1.0	13.2	62.2	251.4
10	1.0	1.3	1.0	1.0
100	1.0	1.0	1.0	1.0

Table 2 Percentage reduction of growth rate and inhibition of yield during the combined limit/range-finding test

Loading rate [redacted] (oil free) WAF (mg/l)	Mean growth rate		Yield (0-72 h)	
	µ (0-72 h)	Reduction (%)	x10^4 cells/ml	Inhibition (%)
Control	0.07707		256.64	
1.0	0.07676	0.4	250.38	2.4
10	0.00000	100.0	0.00	100.0
100	0.00000	100.0	0.00	100.0

7.2. First main test

7.2.1. Measured test substance concentrations

The results of analysis of the samples taken during the 1st main test are described in Table 3 of the appended Analytical Report.

Analyses of the samples taken at the start of the test showed measured concentrations of 27.9, 0.35, 70.9, 0.89 and 275 µg/l for the WAFs prepared at 1.0, 1.8, 3.2, 5.6 and 10 mg/l, respectively. Hence, there was no relationship with the initial loading rates prepared. The highly variable concentrations measured are attributed to the very poor solubility of the UVCB test substance and its stickiness (viscous liquid). Measured concentrations generally decreased during the test period but also some fluctuations were observed in measurements after 24 and 72 hours indicating that test solutions were likely inhomogeneous. Note that at the end of the test high variations in algal cell density were observed in individual replicates of the WAFs prepared at 3.2 and 5.6 mg/l. Consequently, some of the replicates from these groups were individually sampled (replicates not pooled). The average exposure

concentrations calculated for individual replicates were however only marginally different and therefore it was decided to base results on group TWA concentrations. These corresponded to 1.9, 0.38, 2.9, 0.22 and 8.5 µg/l for the WAFs prepared at 1.0, 1.8, 3.2, 5.6 and 10 mg/l (see Table 3).

Table 3 Measured concentrations versus loading rates

Loading rate [redacted] (oil free) WAF (mg/l)	Replicate number	Measured t=0 h (µg/l)	Measured t=24 h (µg/l)	Measured t=72 h (µg/l)	Replicate TWA concentration (µg/l)	Group TWA concentration (µg/l)
1.0	1,2,3	27.9	0.57	1.36	1.9	1.9
1.8	1,2,3	0.35	0.98	0.076	0.38	0.38
3.2	1,2	70.9	0.48	2.96	2.7	2.9
	3			9.35	3.4	
5.6	1	0.89	0.097	0.45	0.24	0.22
	2			0.43	0.23	
	3			0.18	0.19	
10	1,2,3	275	0.95	22.5	8.5	8.5

7.2.2. Mean cell densities

Table 4 shows mean cell densities measured at 24-hour intervals at the different concentrations of [redacted] (oil free). The respective growth curves are shown in Figure 1 (see APPENDIX 1 for the cell densities per replicate).

Table 4 Mean cell densities (x 10^4 cells/ml) during the 1st main test

Test group [redacted] (oil free) TWA (µg/l)	Exposure time (hours)			
	0	24	48	72
Control	1.0	4.2	19.4	86.8
1.9	1.0	2.3	9.4	35.4
0.38	1.0	4.1	19.0	86.5
2.9	1.0	2.0	6.5	21.2
0.22	1.0	3.5	13.4	59.2
8.5	1.0	1.0	1.0	1.0

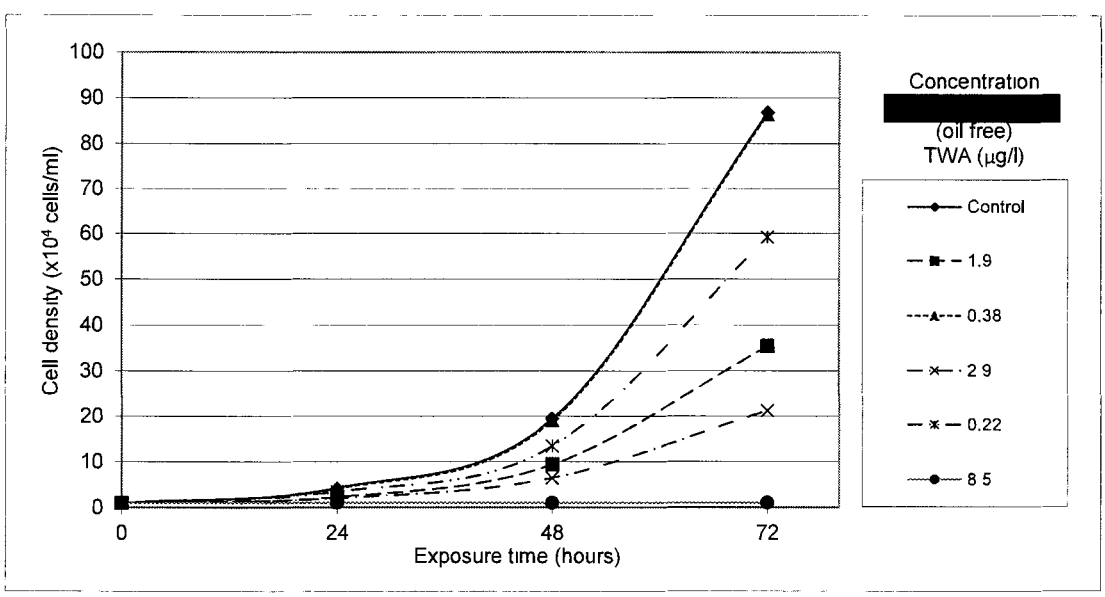


Figure 1 Growth curves at different TWA concentrations of [redacted] (oil free)

7.2.3. Reduction of growth rate and inhibition of yield

Table 5 shows the calculation of the percentages of growth rate reduction (total test period) and the percentages of yield inhibition. Table 6 shows the calculation of the percentages of growth rate reduction at different time intervals (see APPENDIX 1 for the values of growth rate and yield per replicate). Statistical analysis of the data is shown in APPENDIX 2.

Growth rates were generally in the range of the controls at TWA concentrations of 0.22 and 0.38 µg/l during the 72-hour test period (reduction ≤10%), whereas the growth rate of algae exposed to TWA concentrations of 1.9 µg/l and higher were significantly reduced (>20%).
Statistically significant reduction of growth rate was nevertheless found at TWA concentrations of 2.9 µg/l and higher (Bonferroni t test, $\alpha = 0.05$). However, as a biologically significant reduction of 21% was observed at 1.9 µg/l it was decided to set the NOEC at 0.38 µg/l.

Inhibition of yield was below 50% at TWA concentration of 0.22 and 0.38 µg/l, whereas 60% or more inhibition was observed at TWA concentrations of 1.9 µg/l and higher.
Statistically significant inhibition of yield was found at TWA concentrations of 1.9 µg/l and higher (Bonferroni t test, $\alpha = 0.05$). Hence, the NOEC was 0.38 µg/l.

Microscopic observations at the end of the test revealed a normal and healthy appearance of the exposed cells when compared to the control.

Table 5 Percentage reduction of growth rate (total test period) and percentage inhibition of yield during the 1st main test

Test group [redacted] (oil free) TWA (µg/l)	Mean growth rate		Yield (0-72 h)	
	µ (0-72 h)	Reduction (%)	x10^4 cells/ml	Inhibition (%)
Control	0.06193		85.85	
1.9	0.04913	20.7	34.45	59.9
0.38	0.06191	0.0	85.45	0.5
2.9	0.02126	65.7	20.22	76.4
0.22	0.05536	10.6	58.17	32.2
8.5	0.00000	100.0	0.00	100.0

Table 6 Percentage reduction of growth rate at different time intervals during the 1st main test

Test group [redacted] (oil free) TWA (µg/l)	Mean growth rate					
	µ (0-24 h)	Reduction (%)	µ (24-48 h)	Reduction (%)	µ (48-72 h)	Reduction (%)
Control	0.06004		0.06338		0.06236	
1.9	0.03450	42.5	0.05792	8.6	0.05496	11.9
0.38	0.05888	1.9	0.06370	-0.5	0.06316	-1.3
2.9	0.02212	63.2	0.02889	54.4	0.01862	70.1
0.22	0.05092	15.2	0.05430	14.3	0.06086	2.4
8.5	0.00107	98.2	0.00000	100.0	0.00000	100.0

7.3. Second main test

As a consequence of the fact that there was no correlation between the loading rates and the measured concentrations and moreover, the individual replicates showed a rather variable algal growth, it was decided to repeat the algae study with the same test set-up to determine if this was possibly related to handling errors or an inherent solubility characteristic of the test substance.

7.3.1. Measured test substance concentrations

The results of analysis of the samples taken during the 2nd main test are described in Table 4 of the appended Analytical Report.

Analyses of the samples taken at the start of the test showed measured concentrations of 33.1, 0.44, 51.6, 17.7 and 15.2 µg/l for the WAFs prepared at 1.0, 1.8, 3.2, 5.6 and 10 mg/l, respectively. Hence, as was observed in the first main test, there was no relationship with the initial loading rates prepared. The very poor solubility and the stickiness of the viscous liquid UVCB test substance were considered responsible. Measured concentrations generally decreased during the test period but also some fluctuations were observed in measurements after 24 and 72 hours indicating that test solutions were likely inhomogeneous. Note that at the end of the test high variations in algal cell density were observed in individual replicates of the WAF prepared at 3.2 mg/l. Consequently, one replicate of this group was individually sampled (replicates not pooled). The average exposure concentration calculated for this individual replicate was however only marginally different and therefore it was decided to base results on group TWA concentrations. These corresponded to 4.4, 0.15, 4.5, 0.98 and 1.9 µg/l for the WAFs prepared at 1.0, 1.8, 3.2, 5.6 and 10 mg/l (see Table 7).

Table 7 Measured concentrations versus loading rates

Loading rate [redacted] (oil free) WAF (mg/l)	Replicate number	Measured t=0 h (µg/l)	Measured t=24 h (µg/l)	Measured t=72 h (µg/l)	Replicate TWA concentration (µg/l)	Group TWA concentration (µg/l)
1.0	1,2,3	33.1	2.53	1.7	4.4	4.4
1.8	1,2,3	0.44	0.094	0.177	0.15	0.15
3.2	1,2	51.6	2.03	2.19	4.8	4.5
	3			0.376	4.0	
5.6	1,2,3	17.7	0.3	0.33	0.98	0.98
10	1,2,3	15.2	1.17	0.447	1.9	1.9

7.3.2. Mean cell densities

Table 8 shows mean cell densities measured at 24-hour intervals at the different concentrations of [redacted] (oil free). The respective growth curves are shown in Figure 2 (see APPENDIX 1 for the cell densities per replicate).

Table 8 Mean cell densities (x 10^4 cells/ml) during the 2nd main test

Test group [redacted] (oil free) TWA (µg/l)	Exposure time (hours)			
	0	24	48	72
Control	1.0	4.7	18.1	80.9
4.4	1.0	1.0	1.3	2.9
0.15	1.0	3.3	13.3	66.2
4.5	1.0	1.3	2.7	10.9
0.98	1.0	1.6	2.5	6.3
1.9	1.0	1.0	1.0	1.9

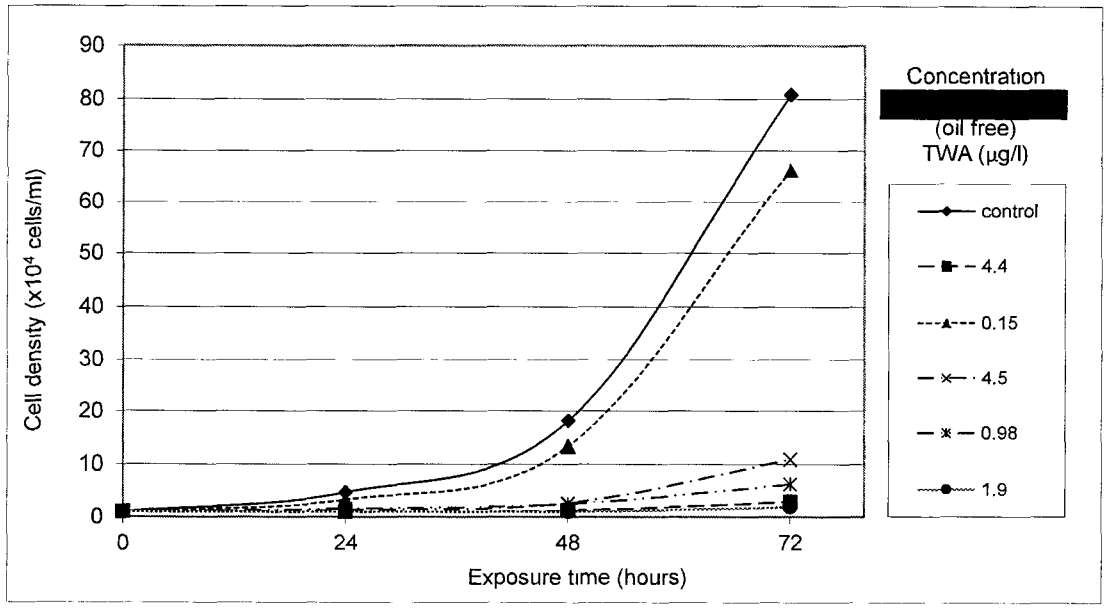


Figure 2 **Growth curves at different TWA concentrations of [redacted] (oil free)**

7.3.3. Reduction of growth rate and inhibition of yield

Table 9 shows the calculation of the percentages of growth rate reduction (total test period) and the percentages of yield inhibition. Table 10 shows the calculation of the percentages of growth rate reduction at different time intervals (see APPENDIX 1 for the values of growth rate and yield per replicate). Statistical analysis of the data is shown in APPENDIX 2.

Growth rate was in the range of the control at a TWA concentration of 0.15 µg/l during the 72-hour test period, whereas the growth rates of algae exposed to 0.98 µg/l and higher were all significantly reduced.
Statistically significant reduction of growth rate was found at TWA concentrations of 0.98 µg/l and higher (Bonferroni t test, $\alpha = 0.05$). The NOEC was 0.15 µg/l.

Yield was inhibited by 18% at the lowest TWA concentration of 0.15 mg/l. At the higher TWA concentrations of 0.98 µg/l and higher yield was inhibited by 88% or more.
Statistically significant inhibition of yield was found at TWA concentrations of 0.98 µg/l and higher (Bonferroni t test, $\alpha = 0.05$). The NOEC was 0.15 µg/l.

Microscopic observations at the end of the test revealed a normal and healthy appearance of the exposed cells when compared to the control.

Table 9 **Percentage reduction of growth rate (total test period) and percentage inhibition of yield during the 2nd main test**

Test group [redacted] (oil free) TWA (µg/l)	Mean growth rate		Yield (0-72 h)	
	µ (0-72 h)	Reduction (%)	x10⁴ cells/ml	Inhibition (%)
Control	0 06095		79.88	
4.4	0.01467	75.9	1.92	97.6
0.15	0.05814	4.6	65.17	18.4
4.5	0.01586	74.0	9.92	87.6
0.98	0.02518	58.7	5.25	93.4
1.9	0.00789	87.1	0.92	98.9

Table 10 Percentage reduction of growth rate at different time intervals during the 2nd main test

Test group [redacted] (oil free) TWA (µg/l)	Mean growth rate					
	µ (0-24 h)	Reduction (%)	µ (24-48 h)	Reduction (%)	µ (48-72 h)	Reduction (%)
Control	0.06369		0.05655		0.06261	
4.4	0.00000	100.0	0.00873	84.6	0.03527	43.7
0.15	0.04944	22.4	0.05799	-2.6	0.06699	-7.0
4.5	0.00963	84.9	0.01526	73.0	0.02270	63.7
0.98	0.01405	77.9	0.02303	59.3	0.03845	38.6
1.9	0.00000	100.0	0.00000	100.0	0.02368	62.2

7.3.4. Determination of effect concentrations

Table 11 shows the effect parameters based on the results of the two main studies performed. Effect parameters are expressed in TWA concentrations, see also APPENDIX 3.

Table 11 Effect parameters

Parameter	Concentration [redacted] (oil free) TWA (µg/l)	95%-confidence interval (µg/l)	Concentration [redacted] (oil free) TWA (µg/l)	95%-confidence interval (µg/l)
	1st main test		*2nd main test*	
NOE_RC	0.38		0.15	
72h-E_RC_{50}	2.3	2.0-2.6	0.88*	0.08-9.5
NOE_YC	0.38		0.15	
72h-E_YC_{50}	1.1*	0.09-14	0.36	0.17-0.76

* Considered estimates based on the broad confidence interval

7.3.5. Experimental conditions

Table 12 shows the pH recorded at the beginning and the end of the test. The pH was within the limits prescribed by the protocol (6.0-9.0, preferably not varying by more than 1.5 unit). The temperature of the test medium was 22.4°C at the start of the 1st main test and 21.1°C at the start of the 2nd main test. During the exposure period of the two studies the temperature measured in the incubator was maintained between 21.4 and 23.1°C. Temperature remained within the limits prescribed by the protocol (21-24°C, constant within 2°C).

Table 12 pH levels recorded during the two main tests

Test group [redacted] (oil free) TWA (µg/l)	Exposure time (hours)	
	0	72
1st main test		
Control	8.0	7.9
1.9	8.1	7.9
0.38	8.0	7.9
2.9	8.0	7.9
0.22	8.0	7.9
8.5	8.0	7.9
2nd main test		
Control	8.1	8.1
4.4	8.2	8.0
0.15	8.2	8.1
4.5	8.2	8.0
0.98	8.2	8.0
1.9	8.1	8.0

8. CONCLUSION

Under the conditions of the present study with *Pseudokirchneriella subcapitata* exposed to various [redacted] (oil free) concentrations, the following toxicity parameters were determined:

The EC_{50} for growth rate reduction (E_RC_{50}: 0-72h) was estimated to correspond to 0.88 µg/l.

The EC_{50} for yield inhibition (E_YC_{50}: 0-72h) was 0.36 µg/l with a 95% confidence interval ranging from 0.17 to 0.76 µg/l.

The NOEC based on TWA concentrations for both growth rate reduction and yield inhibition was 0.15 µg/l. This NOEC is derived from a loading rate (nominal test concentration) of 1.8 mg/l.

Note that results were based on two main studies and that a worst-case approach was followed to determine the EC and NOEC values.

APPENDIX 1 WORKSHEET DATA

1st main test

Table 13 Individual cell densities

Number of inoculated cells at t=0: 1 x10^4 cells/ml					
Test group [redacted] (oil free) TWA (µg/l)	Vessel number	Exposure time (hours)			
		0	24	48	72
Control	1	1 00	4.15	20 25	89.60
	2	1 00	4.30	19.68	90.59
	3	1.00	4.05	17.71	76 30
	4	1.00	4.28	18.10	76.29
	5	1 00	4 59	21.76	102.69
	6	1.00	4 02	18.80	85 63
1.9	1	1 00	2.21	7 64	25.84
	2	1 00	2.04	8.39	33.26
	3	1.00	2.65	12.12	47.25
0.38	1	1.00	3.99	19.57	90.78
	2	1 00	4.08	19 78	89.61
	3	1.00	4 26	17.57	78.97
2.9	1	1.00	1 00	1.00	1.00
	2	1.00	1 28	2.46	1 62
	3	1 00	3.85	15.97	61.04
0.22	1	1.00	2.78	8.39	32.35
	2	1.00	4 30	20.45	93 71
	3	1.00	3.27	11 37	51 44
8.5	1	1 00	1.00	1.00	1.00
	2	1.00	1.08	1.00	1.00
	3	1.00	1 00	1.00	1.00

APPENDIX 1 WORKSHEET DATA – continued –

1st main test

Table 14 Calculation of growth rate and yield

Test group [redacted] (oil free) TWA (µg/l)	Vessel number	Growth rate (µ) 0-72 h	Yield (x10⁴ cells/ml) 0-72 h	Growth rate red. (%) 0-72 h	Yield inhib. (%) 0-72 h
Control	1	0.06243	88.60		
	2	0.06259	89.59		
	3	0.06020	75 30		
	4	0 06020	75 29		
	5	0.06433	101 69		
	6	0.06181	84.63		
	mean	0.06193	85.85		
	CV	3%			
1.9	1	0.04517	24.84	27	71
	2	0 04867	32.26	21	62
	3	0 05355	46 25	14	46
0 38	1	0.06262	89 78	-1	-5
	2	0.06244	88.61	-1	-3
	3	0.06068	77.97	2	9
2.9	1	0.00000	0.00	100	100
	2	0.00668	0.62	89	99
	3	0 05710	60.04	8	30
0.22	1	0.04829	31 35	22	63
	2	0.06306	92.71	-2	-8
	3	0.05473	50.44	12	41
8.5	1	0.00000	0.00	100	100
	2	0.00000	0.00	100	100
	3	0 00000	0.00	100	100

APPENDIX 1 WORKSHEET DATA – continued –

1st main test

Table 15 Calculation of growth rate (section-by-section)

Test group [redacted] (oil free) TWA (µg/l)	Vessel number	Growth rate (µ)			Growth rate reduction (%)		
		0-24 h	24-48 h	48-72 h	0-24 h	24-48 h	48-72 h
Control	1	0 05930	0 06604	0.06197			
	2	0.06078	0.06338	0 06361			
	3	0.05823	0 06153	0.06085			
	4	0.06053	0.06013	0.05994			
	5	0.06348	0.06486	0.06465			
	6	0.05792	0 06433	0 06316			
	mean	0 06004	0.06338	0.06236			
	CV	3%	3%	3%			
	The mean CV for section-by-section specific growth rate was: 4%						
1.9	1	0.03306	0.05164	0 05079	45	19	19
	2	0.02979	0 05881	0.05741	50	7	8
	3	0 04064	0.06332	0.05669	32	0	9
0 38	1	0.05761	0.06632	0.06393	4	-5	-3
	2	0.05861	0.06576	0 06295	2	-4	-1
	3	0 06042	0.05902	0.06261	-1	7	0
2.9	1	0.00000	0.00000	0 00000	100	100	100
	2	0.01022	0.02735	0 00000	83	57	100
	3	0 05614	0.05932	0.05586	6	6	10
0 22	1	0.04259	0.04603	0.05624	29	27	10
	2	0.06081	0 06493	0 06343	-1	-2	-2
	3	0 04937	0 05192	0.06290	18	18	-1
8.5	1	0.00000	0.00000	0 00000	100	100	100
	2	0.00321	0.00000	0.00000	95	100	100
	3	0 00000	0 00000	0.00000	100	100	100

APPENDIX 1 WORKSHEET DATA – continued –

2nd main test

Table 16 Individual cell densities

Number of inoculated cells at t=0: 1 x10^4 cells/ml					
Test group [redacted] (oil free) TWA (µg/l)	Vessel number	Exposure time (hours)			
		0	24	48	72
Control	1	1.00	3.00	16.50	72.75
	2	1.00	4.75	18.50	83.00
	3	1.00	5.50	14.00	84.25
	4	1.00	5 50	18.50	71.00
	5	1 00	4 25	18.00	94.25
	6	1.00	5 25	23.25	80.00
4.4	1	1.00	1.00	1 25	3.25
	2	1.00	1.00	1 50	3.25
	3	1.00	1.00	1 00	2.25
0 15	1	1.00	3.75	16.00	67.75
	2	1 00	2 50	13.00	74.00
	3	1.00	3 75	11 00	56.75
4.5	1	1.00	1 00	1.00	1.00
	2	1.00	1.00	1.00	1 00
	3	1.00	2.00	6 00	30.75
0.98	1	1.00	2.75	2 75	5.75
	2	1.00	1.00	3 00	8.00
	3	1.00	1.00	1.75	5.00
1.9	1	1 00	1.00	1.00	2.00
	2	1.00	1.00	1.00	2.75
	3	1 00	1 00	1.00	1.00

APPENDIX 1 WORKSHEET DATA – continued –

2nd main test

Table 17 Calculation of growth rate and yield

Test group [redacted] (oil free) TWA (µg/l)	Vessel number	Growth rate (µ) 0-72 h	Yield (x10⁴ cells/ml) 0-72 h	Growth rate red. (%) 0-72 h	Yield inhib. (%) 0-72 h
Control	1	0 05954	71.75		
	2	0.06137	82.00		
	3	0.06158	83 25		
	4	0.05920	70 00		
	5	0 06314	93.25		
	6	0.06086	79 00		
	mean	0.06095	79.88		
	CV	2%			
4.4	1	0.01637	2.25	73	97
	2	0.01637	2.25	73	97
	3	0.01126	1.25	82	98
0.15	1	0 05855	66.75	4	16
	2	0.05978	73.00	2	9
	3	0.05609	55.75	8	30
4 5	1	0.00000	0.00	100	100
	2	0.00000	0.00	100	100
	3	0 04758	29.75	22	63
0 98	1	0.02429	4 75	60	94
	2	0.02888	7.00	53	91
	3	0 02235	4.00	63	95
1.9	1	0.00963	1.00	84	99
	2	0.01405	1.75	77	98
	3	0.00000	0 00	100	100

APPENDIX 1 WORKSHEET DATA – continued –

2nd main test

Table 18 Calculation of growth rate (section-by-section)

Test group [redacted] (oil free) TWA (µg/l)	Vessel number	Growth rate (µ)			Growth rate reduction (%)		
		0-24 h	24-48 h	48-72 h	0-24 h	24-48 h	48-72 h
Control	1	0.04578	0 07103	0.06182			
	2	0.06492	0.05665	0.06254			
	3	0.07103	0 03893	0.07478			
	4	0.07103	0.05054	0.05604			
	5	0.06029	0.06014	0.06898			
	6	0.06909	0.06200	0.05149			
	mean	0.06369	0 05655	0 06261			
	CV	15%	19%	13%			
	The mean CV for section-by-section specific growth rate was 16%						
4.4	1	0.00000	0.00930	0.03981	100	84	36
	2	0 00000	0.01689	0.03222	100	70	49
	3	0 00000	0.00000	0.03379	100	100	46
0.15	1	0 05507	0.06045	0.06013	14	-7	4
	2	0 03818	0.06869	0.07246	40	-21	-16
	3	0.05507	0.04484	0 06837	14	21	-9
4.5	1	0.00000	0.00000	0 00000	100	100	100
	2	0.00000	0.00000	0.00000	100	100	100
	3	0 02888	0.04578	0 06809	55	19	-9
0.98	1	0 04215	0.00000	0.03073	34	100	51
	2	0.00000	0.04578	0.04087	100	19	35
	3	0.00000	0.02332	0.04374	100	59	30
1.9	1	0 00000	0.00000	0.02888	100	100	54
	2	0.00000	0.00000	0 04215	100	100	33
	3	0.00000	0.00000	0.00000	100	100	100

APPENDIX 2 STATISTICS: GROWTH RATE (0-72 HOURS)

1st main test:

Chi-Square Test for Normality

	Actual and Expected Frequencies				
INTERVAL	<-1.5	-1.5 to <-0.5	-0.5 to 0.5	>0.5 to 1.5	>1.5
EXPECTED	1.2060	4.3560	6.8760	4.3560	1.2060
OBSERVED	0	6	7	4	1

Chi-Square = 1.8930 (p-value = 0.7554)

Critical Chi-Square = 13.277 (alpha = 0.01 , df = 4)
= 9.488 (alpha = 0.05 , df = 4)

Data PASS normality test (alpha = 0.01). Continue analysis.

Levene's Test for Homogeneity of Variance
ANOVA Table

SOURCE	DF	SS	MS	F
Between	4	0.0007	0.0002	1.5643
Within (Error)	13	0.0016	0.0001	
Total	17	0.0023		

(p-value = 0.2420)

Critical F = 5.2053 (alpha = 0.01, df = 4,13)
= 3.1791 (alpha = 0.05, df = 4,13)

Since F < Critical F FAIL TO REJECT Ho: All equal (alpha = 0.01)

ANOVA Table

SOURCE	DF	SS	MS	F
Between	4	0.0038	0.0009	5.8243
Within (Error)	13	0.0021	0.0002	
Total	17	0.0059		

(p-value = 0.0065)

Critical F = 5.2053 (alpha = 0.01, df = 4,13)
= 3.1791 (alpha = 0.05, df = 4,13)

Since F > Critical F REJECT Ho: All equal (alpha = 0.05)

Bonferroni t-Test - TABLE 1 OF 2 Ho: Control<Treatment

GROUP	IDENTIFICATION	TRANSFORMED MEAN	MEAN CALCULATED IN ORIGINAL UNITS	t STAT	SIG 0.05
1	Control	0.0619	0.0619		
2	1.9	0.0491	0.0491	1.4209	
3	0.38	0.0619	0.0619	0.0015	
4	2.9	0.0213	0.0213	4.5154	*
5	0.22	0.0554	0.0554	0.7291	

Bonferroni t critical value = 2.5326 (1 Tailed, alpha = 0.05, df = 4,13)

Bonferroni t-Test - TABLE 2 OF 2 Ho: Control<Treatment

GROUP	IDENTIFICATION	NUM OF REPS	MIN SIG DIFF (IN ORIG. UNITS)	% OF CONTROL	DIFFERENCE FROM CONTROL
1	Control	6			
2	1.9	3	0.0228	36.8	0.0128
3	0.38	3	0.0228	36.8	0.0000
4	2.9	3	0.0228	36.8	0.0407
5	0.22	3	0.0228	36.8	0.0066

APPENDIX 2 STATISTICS: GROWTH RATE (0-72 HOURS)

2nd main test:

Chi-Square Test for Normality

INTERVAL	<-1.5	-1.5 to <-0.5	-0.5 to 0.5	>0.5 to 1.5	>1.5
EXPECTED	1.4070	5.0820	8.0220	5.0820	1.4070
OBSERVED	0	8	6	6	1

Chi-Square = 3.8757 (p-value = 0.4231)
Critical Chi-Square = 13.277 (alpha = 0.01 , df = 4)
= 9.488 (alpha = 0.05 , df = 4)

Data PASS normality test (alpha = 0.01). Continue analysis.

Levene's Test for Homogeneity of Variance
ANOVA Table

SOURCE	DF	SS	MS	F
Between	5	0.0005	0.0001	0.9871
Within (Error)	15	0.0016	0.0001	
Total	20	0.0021		

(p-value = 0.4577)

Critical F = 4.5556 (alpha = 0.01, df = 5,15)
= 2.9013 (alpha = 0.05, df = 5,15)
Since F < Critical F FAIL TO REJECT Ho: All equal (alpha = 0.01)

ANOVA Table

SOURCE	DF	SS	MS	F
Between	5	0.0105	0.0021	18.8259
Within (Error)	15	0.0017	0.0001	
Total	20	0.0121		

(p-value = 0.0000)

Critical F = 4.5556 (alpha = 0.01, df = 5,15)
= 2.9013 (alpha = 0.05, df = 5,15)

Since F > Critical F REJECT Ho: All equal (alpha = 0.05)

Bonferroni t-Test - TABLE 1 OF 2 Ho: Control<Treatment

GROUP	IDENTIFICATION	TRANSFORMED MEAN	MEAN CALCULATED IN ORIGINAL UNITS	t STAT	SIG 0.05
1	control	0.0609	0.0609		
2	4.4	0.0147	0.0147	6.2034	*
3	0.15	0.0581	0.0581	0.3764	
4	4.5	0.0159	0.0159	6.0435	*
5	0.98	0.0252	0.0252	4.7951	*
6	1.9	0.0079	0.0079	7.1113	*

Bonferroni t critical value = 2.6025 (1 Tailed, alpha = 0.05, df = 5,15)

Bonferroni t-Test - TABLE 2 OF 2 Ho: Control<Treatment

GROUP	IDENTIFICATION	NUM OF REPS	MIN SIG DIFF (IN ORIG. UNITS)	% OF CONTROL	DIFFERENCE FROM CONTROL
1	control	6			
2	4.4	3	0.0194	31.9	0.0463
3	0.15	3	0.0194	31.9	0.0028
4	4.5	3	0.0194	31.9	0.0451
5	0.98	3	0.0194	31.9	0.0358
6	1.9	3	0.0194	31.9	0.0531

APPENDIX 2 STATISTICS: YIELD (0-72 HOURS)

1st main test:

Chi-Square Test for Normality

INTERVAL	<-1.5	-1.5 to <-0.5	-0.5 to 0.5	>0.5 to 1.5	>1.5
	Actual and Expected Frequencies				
EXPECTED	1.2060	4.3560	6.8760	4.3560	1.2060
OBSERVED	0	7	6	4	1

Chi-Square = 2.9867 (p-value = 0.5600)

Critical Chi-Square = 13.277 (alpha = 0.01 , df = 4)
= 9.488 (alpha = 0.05 , df = 4)

Data PASS normality test (alpha = 0.01). Continue analysis.

Levene's Test for Homogeneity of Variance
ANOVA Table

SOURCE	DF	SS	MS	F
Between	4	782.0734	195.5183	0.7141
Within (Error)	13	3559.6039	273.8157	
Total	17	4341.6773		

(p-value = 0.5970)

Critical F = 5.2053 (alpha = 0.01, df = 4,13)
= 3.1791 (alpha = 0.05, df = 4,13)

Since F < Critical F FAIL TO REJECT Ho: All equal (alpha = 0.01)

ANOVA Table

SOURCE	DF	SS	MS	F
Between	4	12618.8875	3154.7219	7.9348
Within (Error)	13	5168.5509	397.5808	
Total	17	17787.4384		

(p-value = 0.0018)

Critical F = 5.2053 (alpha = 0.01, df = 4,13)
= 3.1791 (alpha = 0.05, df = 4,13)

Since F > Critical F REJECT Ho: All equal (alpha = 0.05)

Bonferroni t-Test - TABLE 1 OF 2 Ho: Control<Treatment

GROUP	IDENTIFICATION	TRANSFORMED MEAN	MEAN CALCULATED IN ORIGINAL UNITS	t STAT	SIG 0.05
1	Control	85.8500	85.8500		
2	1.9	34.4500	34.4500	3.6456	*
3	0.38	85.4533	85.4533	0.0281	
4	2.9	20.2200	20.2200	4.6548	*
5	0.22	58.1667	58.1667	1.9635	

Bonferroni t critical value = 2.5326 (1 Tailed, alpha = 0.05, df = 4,13)

Bonferroni t-Test - TABLE 2 OF 2 Ho: Control<Treatment

GROUP	IDENTIFICATION	NUM OF REPS	MIN SIG DIFF (IN ORIG. UNITS)	% OF CONTROL	DIFFERENCE FROM CONTROL
1	Control	6			
2	1.9	3	35.7084	41.6	51.4000
3	0.38	3	35.7084	41.6	0.3967
4	2.9	3	35.7084	41.6	65.6300
5	0.22	3	35.7084	41.6	27.6833

APPENDIX 2 STATISTICS: YIELD (0-72 HOURS)

2nd main test:

Chi-Square Test for Normality

Actual and Expected Frequencies

INTERVAL	<-1.5	-1.5 to <-0.5	-0.5 to 0.5	>0.5 to 1.5	>1.5
EXPECTED	1.4070	5.0820	8.0220	5.0820	1.4070
OBSERVED	0	8	6	6	1

Chi-Square = 3.8757 (p-value = 0.4231)

Critical Chi-Square = 13.277 (alpha = 0.01 , df = 4)
= 9.488 (alpha = 0.05 , df = 4)

Data PASS normality test (alpha = 0.01). Continue analysis.

Levene's Test for Homogeneity of Variance
ANOVA Table

SOURCE	DF	SS	MS	F
Between	5	246.0610	49.2122	0.9480
Within (Error)	15	778.6771	51.9118	
Total	20	1024.7381		

(p-value = 0.4790)

Critical F = 4.5556 (alpha = 0.01, df = 5,15)
= 2.9013 (alpha = 0.05, df = 5,15)
Since F < Critical F FAIL TO REJECT Ho: All equal (alpha = 0.01)

ANOVA Table

SOURCE	DF	SS	MS	F
Between	5	26122.0670	5224.4134	70.6791
Within (Error)	15	1108.7604	73.9174	
Total	20	27230.8274		

(p-value = 0.0000)

Critical F = 4.5556 (alpha = 0.01, df = 5,15)
= 2.9013 (alpha = 0.05, df = 5,15)
Since F > Critical F REJECT Ho: All equal (alpha = 0.05)

Bonferroni t-Test - TABLE 1 OF 2 Ho: Control<Treatment

GROUP	IDENTIFICATION	TRANSFORMED MEAN	MEAN CALCULATED IN ORIGINAL UNITS	t STAT	SIG 0.05
1	control	79.8750	79.8750		
2	4.4	1.9167	1.9167	12.8234	*
3	0.15	65.1667	65.1667	2.4194	
4	4.5	9.9167	9.9167	11.5075	*
5	0.98	5.2500	5.2500	12.2751	*
6	1.9	0.9167	0.9167	12.9879	*

Bonferroni t critical value = 2.6025 (1 Tailed, alpha = 0.05, df = 5,15)

Bonferroni t-Test - TABLE 2 OF 2 Ho: Control<Treatment

GROUP	IDENTIFICATION	NUM OF REPS	MIN SIG DIFF (IN ORIG. UNITS)	% OF CONTROL	DIFFERENCE FROM CONTROL
1	control	6			
2	4.4	3	15.8214	19.8	77.9583
3	0.15	3	15.8214	19.8	14.7083
4	4.5	3	15.8214	19.8	69.9583
5	0.98	3	15.8214	19.8	74.6250
6	1.9	3	15.8214	19.8	78.9583

APPENDIX 3 EC-VALUES

1st main test:

Table 19 EC-values for growth rate reduction

Concentration (µg/l)	X Log conc. (µg/l)	Y Reduction (%)
1.9	0 279	27.1
1.9	0 279	21.4
1.9	0.279	13.5
0.38	0.000	-1.1
0.38	0.000	-0.8
0 38	0.000	2 0
2 9	0.462	100 0
2.9	0.462	89.2
2.9	*	7.8
0.22	*	22.0
0.22	*	-1.8
0.22	*	11.6
8.5	*	100 0
8.5	*	100.0
8.5	*	100 0

* Not used for calculation of the EC-values

Slope	402 6067
Intercept	-91.5606
Multiple R	0.9887
n = number of observations	5

Regression line. Y= 402.61 X - 91.56

Prediction of X values based on known Y values

Known Y Reduction (%)	10^{Xreg} (µg/l)	$10^{X95\%-}$ (µg/l)	$10^{X95\%+}$ (µg/l)
10	1.79	1.53	2.09
20	1 89	1.63	2.20
50	2 25	1.95	2.59

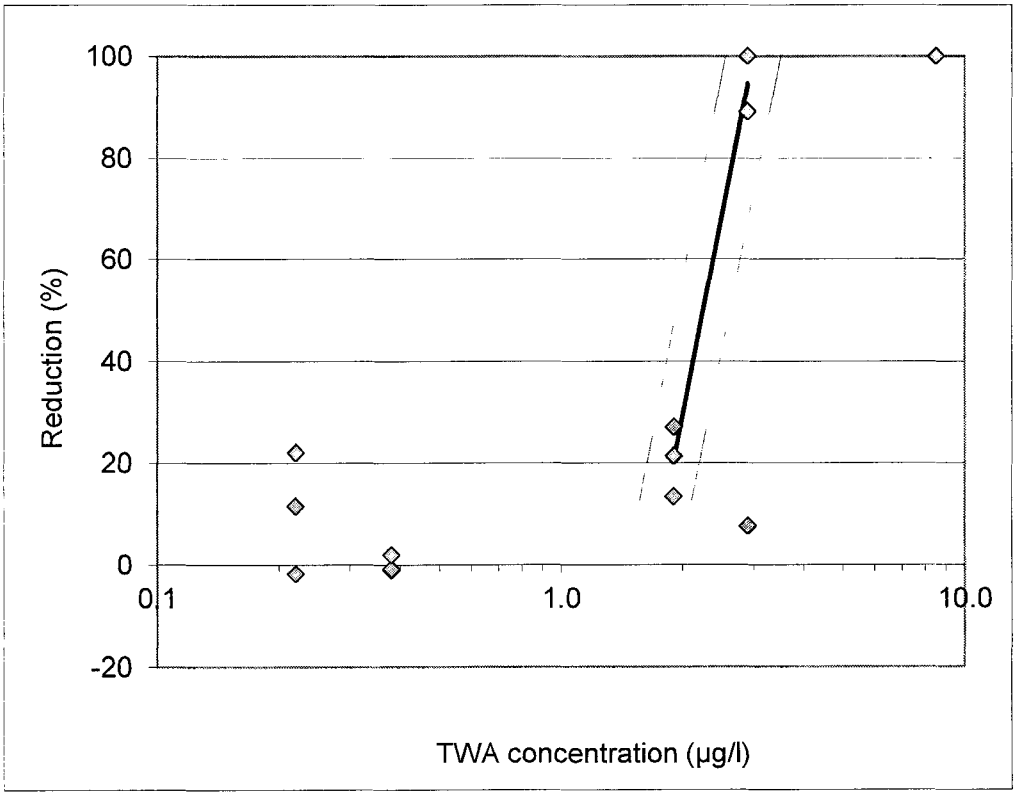


Figure 3 Percentage reduction of growth rate as function of the log TWA concentration (µg/l) of [redacted] (oil free).

Dashed curves represent the 95% confidence limits.

APPENDIX 3 EC-VALUES – continued –

1st main test:

Table 20 EC-values for yield inhibition

Concentration (µg/l)	X Log conc. (µg/l)	Y Inhibition (%)
1.9	0.279	71 1
1.9	0.279	62.4
1.9	0.279	46 1
0.38	-0.420	-4.6
0.38	-0.420	-3 2
0.38	-0.420	9 2
2.9	0 462	100 0
2.9	0.462	99.3
2.9	0.462	30.1
0.22	-0.658	63.5
0.22	-0 658	-8.0
0.22	-0 658	41.2
8.5	0 929	100.0
8.5	0 929	100.0
8 5	0 929	100 0

Slope	54 1256
Intercept	47.3871
Multiple R	0.7824
n = number of observations:	15

Regression line. Y= 54.13 X + 47.39

Prediction of X values based on known Y values			
Known Y Inhibition (%)	10^{Xreg} (µg/l)	$10^{X95\%-}$ (µg/l)	$10^{X95\%+}$ (µg/l)
10	0 20	0.01	3.05
20	0.31	0 02	4.40
50	1 12	0 09	14.43

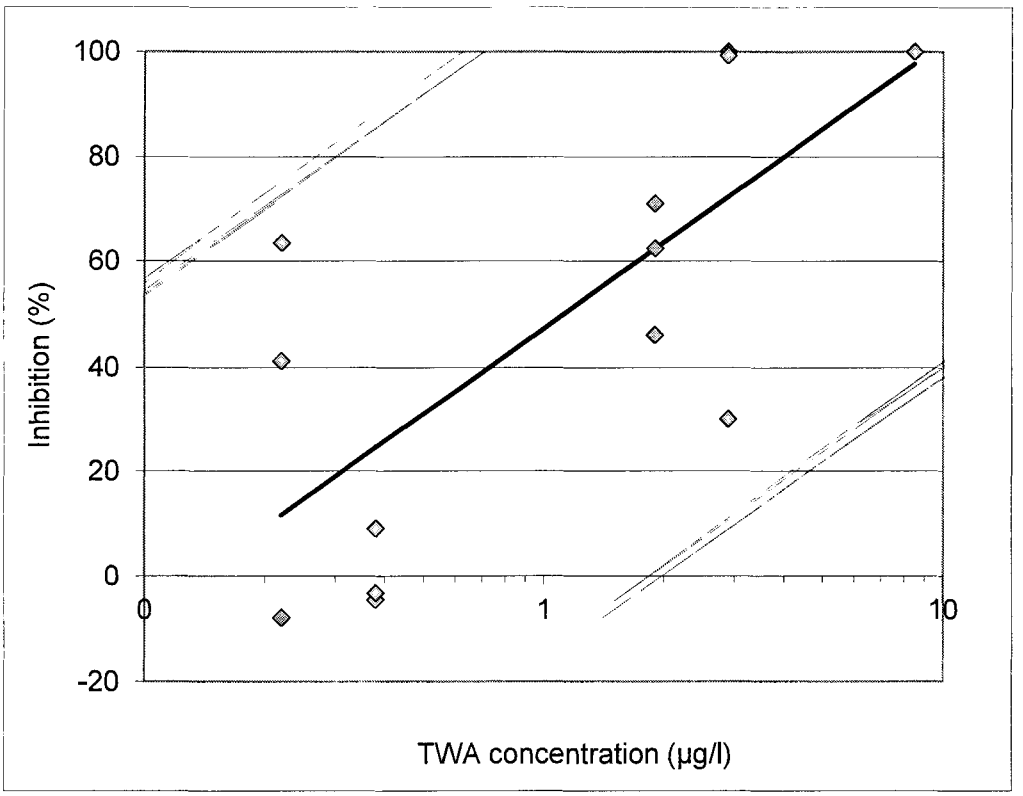


Figure 4 Percentage inhibition of yield as function of the log TWA concentration (µg/l) of [redacted] (oil free).

Dashed curves represent the 95% confidence limits.

APPENDIX 3 EC-VALUES

2nd main test:

Table 21 EC-values for growth rate reduction

Concentration (µg/l)	X Log conc. (µg/l)	Y Reduction (%)
4.4	0.643	73.1
4.4	0.643	73.1
4 4	0 643	81.5
0.15	-0.824	3.9
0.15	-0.824	1 9
0.15	-0.824	8.0
4.5	0.653	100 0
4.5	0.653	100.0
4 5	0.653	21.9
0.98	-0.009	60 1
0 98	-0.009	52.6
0.98	-0.009	63 3
1 9	0.279	84.2
1.9	0.279	76.9
1.9	0 279	100.0

Slope.	48.7793
Intercept	52.8069
Multiple R	0 7851
n = number of observations	15

Regression line Y= 48.78 X + 52.81

Prediction of X values based on known Y values			
Known Y Reduction (%)	**10^{Xreg} (µg/l)**	**$10^{X95\%-}$ (µg/l)**	**$10^{X95\%+}$ (µg/l)**
10	0.13	0.01	1.83
20	0.21	0 02	2.69
50	0 88	0.08	9.51

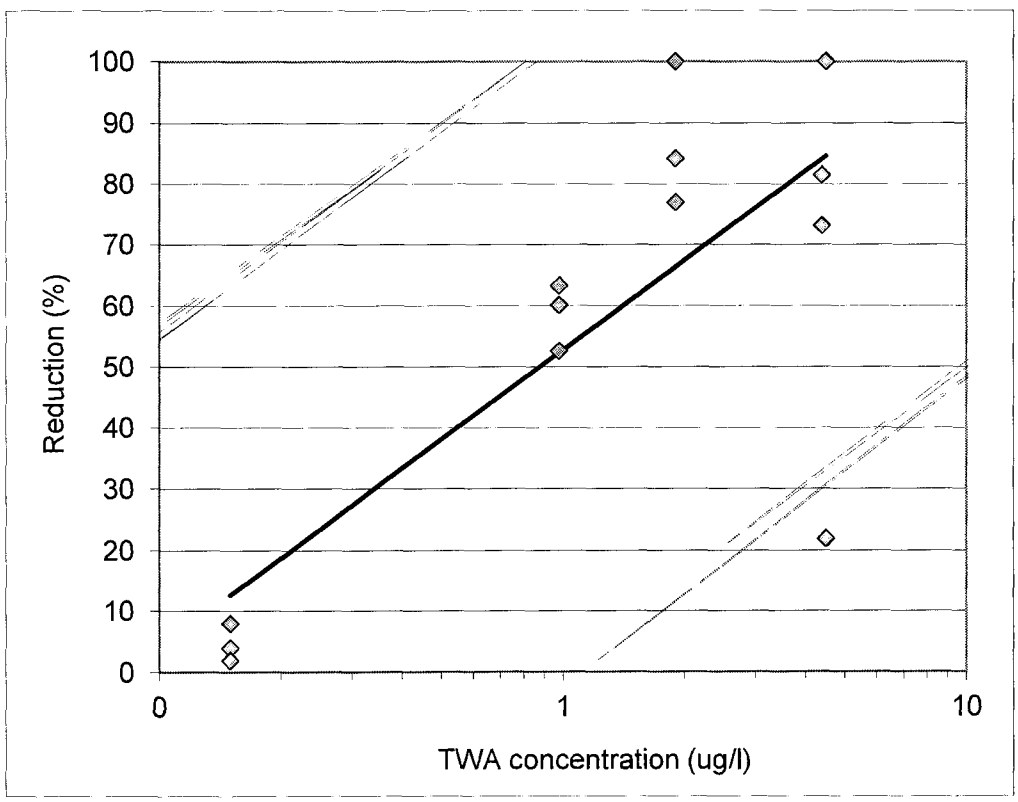


Figure 5 Percentage reduction of growth rate as function of the log TWA concentration (µg/l) of [redacted] (oil free).

Dashed curves represent the 95% confidence limits.

APPENDIX 3 EC-VALUES – continued –

2nd main test:

Table 22 EC-values for yield inhibition

Concentration (µg/l)	X Log conc. (µg/l)	Y Inhibition (%)
4.4	*	97.2
4.4	*	97.2
4.4	*	98.4
0.15	-0.824	16.4
0.15	-0.824	8 6
0.15	-0.824	30 2
4.5	*	100 0
4.5	*	100.0
4.5	*	62.8
0.98	-0.009	94.1
0 98	-0.009	91 2
0.98	-0.009	95 0
1.9	0 279	98.7
1.9	0 279	97.8
1.9	0.279	100.0

* Not used for calculation of the EC-values

Slope	77.1285
Intercept	84.4725
Multiple R	0.9714
n = number of observations	9

Regression line Y= 77.13 X + 84.47

Prediction of X values based on known Y values

Known Y Inhibition (%)	10^{Xreg} (µg/l)	$10^{X95\%-}$ (µg/l)	$10^{X95\%+}$ (µg/l)
10	0.11	0.05	0.25
20	0 15	0.06	0.33
50	0.36	0.17	0.76

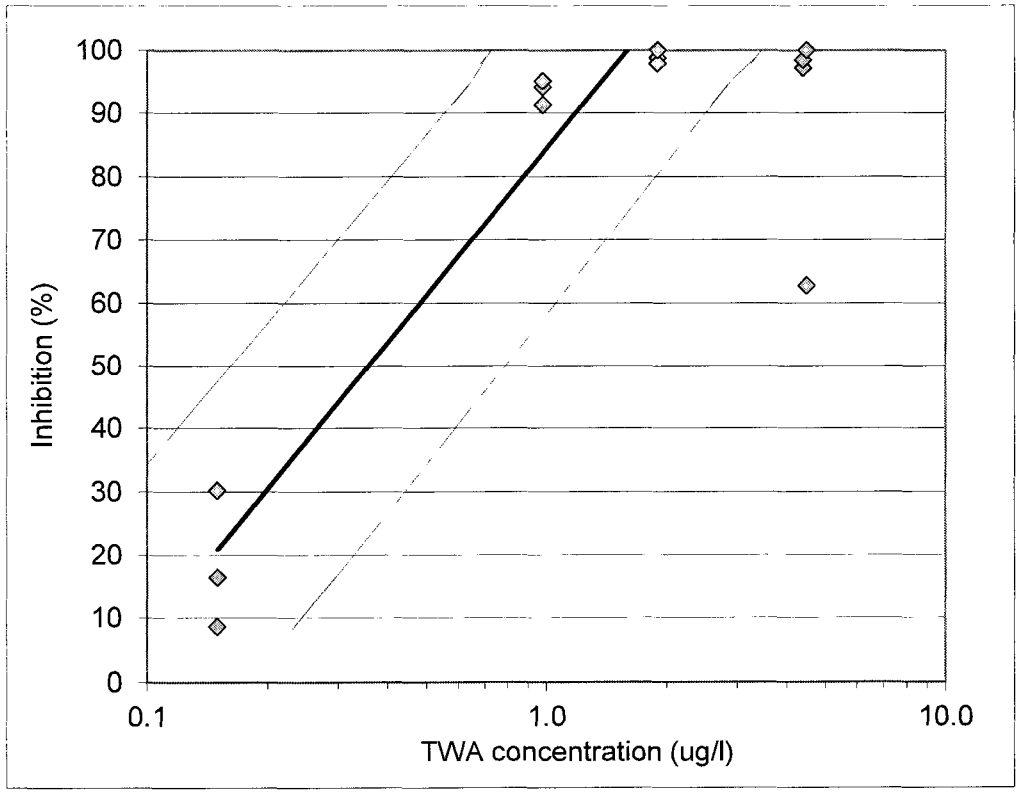


Figure 6 Percentage inhibition of yield as function of the log TWA concentration (µg/l) of [redacted] (oil free).

Dashed curves represent the 95% confidence limits.

APPENDIX 4 REFERENCE TEST

Pseudokirchneriella subcapitata, strain: NIVA CHL-1. Fresh water algal growth inhibition test with potassium dichromate (NOTOX Project 498547).

Start of first exposure: 02 January 2012
Completion last exposure: 05 January 2012

The study procedures described in this report were based on the OECD guideline No. 201, Adopted March 23, 2006 and ISO Standard 8692, Second edition, 01 October 2004.

This reference test was carried out to check the sensitivity of the test system used by NOTOX to Potassium dichromate (Merck, Art. 1.04864, Batch K34869764 607).

Algae were exposed for a period of 72 hours to $K_2Cr_2O_7$ (Potassium dichromate) concentrations of 0.18, 0.32, 0.56, 1.0, 1.8 and 3.2 mg/l and to a control. The initial cell density was 1.0×10^4 cells/ml.

Results:

Overview of % reduction of growth rate and % inhibition of yield in the reference test:

Nominal conc. K2Cr2O7 (mg/l)	Mean growth rate		Yield (0-72 h)	
	μ (0-72 h)	Reduction (%)	$x10^4$ cells/ml	Inhibition (%)
Control	0.07851		284.68	
0.18	0.07807	0.6	275.34	3.3
0.32	0.07707	1.8	256.53	9.9
0.56	0.07220	8.0	181.02	36.4
1.0	0.05811	26.0	64.71	77.3
1.8	0.03514	55.2	11.59	95.9
3.2	0.02622	66.6	5.61	98.0

Potassium dichromate reduced growth rate of this fresh water algae species at nominal concentrations of 0.56 mg/l and higher.

The EC_{50} for growth rate reduction (E_RC_{50}: 0-72h) was 1.8 mg/l with a 95% confidence interval ranging from 1.4 to 2.4 mg/l. The historical ranges for growth rate reduction lie between 0.82 and 2.3 mg/l. Hence, the E_RC_{50}: 0-72h for the algal culture tested corresponds with this range.

The EC_{50} for yield inhibition (E_YC_{50}: 0-72h) was 0.69 mg/l with a 95% confidence interval ranging from 0.50 to 0.95 mg/l. The historical ranges of the 72h-EC_{50} for yield inhibition lie between 0.43 and 1.1 mg/l. Hence, the E_YC_{50}: 0-72h for the algal culture tested corresponds with this range.

The protocol, raw data and report of this study are kept in the NOTOX archives. The test described above was performed under GLP conditions with a QA-check.

APPENDIX 5 ANALYTICAL REPORT

DETERMINATION OF THE CONCENTRATIONS

Author

E. Baltussen, PhD.

1. CONTENTS

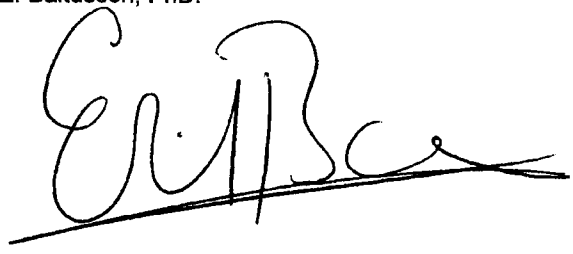
TABLES

2. REPORT APPROVAL

NOTOX B.V.

Principal Scientist
Analytical Chemistry

E. Baltussen, PhD.



Date: 20 August 2012

3. INTRODUCTION

3.1. Preface

Study plan analytical phase
Start : 24 February 2012
Completion : 13 April 2012

3.2. Aim of the study

The purpose of the analytical phase was to determine the actual concentrations in samples taken from the test solutions used during the ecotoxicity test.

4. MATERIALS AND METHODS

4.1. Reagents

Water	Tap water purified by a Milli-Q water purification system (Millipore, Bedford, MA, USA).
Acetonitrile	Biosolve, Valkenswaard, The Netherlands.
Formic acid	Biosolve.
Tetrahydrofuran (THF)	VWR International, Leuven, Belgium.
M2-medium	see main report.

All reagents were of analytical grade, unless specified otherwise.

4.2. Samples

The samples were stored in the freezer (≤ -15°C). Storage stability of samples under these conditions was demonstrated in NOTOX project 498463.

On the day of analysis, the samples were defrosted at room temperature. The test samples were diluted in a 1:3 (v:v) ratio with acetonitrile and analysed. If necessary, the samples were further diluted with 75/25 (v/v) acetonitrile/M2-medium to obtain concentrations within the calibration range.

4.3. Analytical method

4.3.1. Analytical conditions

Quantitative analysis was based on the analytical method validated for the test substance in NOTOX project 498463.

Instrument	Acquity UPLC system (Waters, Milford, MA, USA)
Detector	Xevo TQ-S mass spectrometer (Waters)
Column	Acquity UPLC BEH C18, 100 mm × 2.1 mm i.d., dp = 1.7 µm (Waters)
Column temperature	40°C ± 1°C
Injection volume	5 µl
Mobile phase	0.05% formic acid in 85/15 (v/v) acetonitrile/water
Flow	0.5 ml/min
MS detection	
Ionisation source	ESI^+
Cone voltage	50 V
Collision energy	26
Quantitation	*m/z* 382.3 → *m/z* 200.1

4.3.2. Preparation of the calibration solutions

Stock and spiking solutions
Stock solutions of the test substance were prepared in THF at concentrations of 1020 – 1576 mg/l.

Spiking solutions were made up from a stock solution and/or dilutions of this solution. The solvent of the spiking solutions was THF.

Calibration solutions
Five solutions with the test substance in the concentration range of 0.02 - 3 mg/l were prepared in acetonitrile from two stock solutions. The solutions were 100-times diluted with 75/25 (v/v) acetonitrile/M2-medium to obtain calibration solutions in the concentration range of 0.2 - 30 µg/l.

Procedural recovery samples
1 ml blank medium was spiked with the test substance at a target concentration of 0.01, 1 or 10 mg/l. The accuracy samples were treated similarly as the test samples (see paragraph 4.2 'Samples').

4.3.3. Sample injections

Calibration solutions were injected in duplicate. Test samples and procedural recovery samples were analysed by single injection.

4.4. Electronic data capture

System control, data acquisition and data processing were performed using the following programme:
- MassLynx version 4.1 (Waters, Milford, MA, USA)

Temperature, relative humidity and/or atmospheric pressure during sample storage and/or performance of the studies was monitored continuously using the following programme:
- REES Centron Environmental Monitoring system version SQL 2.0 (REES Scientific, Trenton, NJ, USA).

4.5. Formulas

Response (R)	Peak area test substance [units]
Calibration curve	$R = a\,C_N + b$ where: C_N = nominal concentration [mg/l] a = slope [units × l/mg] b = intercept [units]
Analysed concentration (C_A)	$C_A = \frac{(R-b)}{a} \times d$ [mg/l] where: d = dilution factor
Recovery	$\frac{C_A}{C_N} \times 100$ [%]
Relative to nominal concentration	$\frac{C_A}{C_N} \times 100$ [%]
Relative to initial concentration	$\frac{C_A\,(t = x\,\text{hours})}{C_A\,(t = 0\,\text{hours})} \times 100$ [%]

5. RESULTS

5.1. Calibration curves

Calibration curves were constructed using five concentrations. For each concentration, two responses were used. Linear regression analysis was performed using the least squares method with a 1/concentration^2 weighting factor. The coefficient of correlation (r) was > 0.99 for each curve.

5.2. Samples

5.2.1. Procedural recovery samples

The results for the procedural recovery samples are given in Table 1.

The mean recoveries of the procedural recovery samples fell within the criterion of 70-110%. It demonstrated that the analytical method was adequate for the determination of the test substance in the test samples.

5.2.2. Test samples

The results for the test samples are given in Table 2, Table 3 and Table 4.

6. TABLES

Table 1 Procedural recovery samples

Date of preparation [dd-mm-yy]	Date of analysis [dd-mm-yy]	Target concentration [mg/l]	Nominal concentration [mg/l]	Analysed concentration [mg/l]	Recovery [%]	Mean recovery [%]
24-02-12	24-02-12	0.01	0.0102 0.0102	0.0104 0.0102	102 100	101
24-02-12	24-02-12	10	10.2 10.2	8.69 8.93	85 88	86
11-04-12	11-04-12	0.01	0.00997 0.00997	0.0105 0.0115	105 115	110
11-04-12	11-04-12	1	1.00 1.00	1.09 1.08	109 108	109

Table 2 Concentrations of the test substance in test medium - combined limit/range-finding test

Time of sampling [hours]	Date of sampling [dd-mm-yy]	Date of analysis [1] [dd-mm-yy]	Loading rate [2] [mg/l]	Concentration analysed [mg/l]	Relative to initial [%]
0	06-02-12	24-02-12	1	0.000740[3]	
			10	0.0566	
72	09-02-12	24-02-12	1	0.000715[3]	97
			10	0.00616	11

1 Samples were stored in the freezer (≤ -15°C) until the day of analysis.

2 A water accommodated fraction (WAF) prepared at the loading rate

3 Obtained by extrapolation of the calibration curve.

Table 3 Concentrations of the test substance in test medium – first main study

Time of sampling [hours]	Date of sampling [dd-mm-yy]	Date of analysis [1] [dd-mm-yy]	Loading rate [2] [mg/l]	Concentration analysed [mg/l]	Relative to initial [%]
0	12-03-12	11-04-12	0	0.000150[3]	
			1	0.0279	
			1.8	0.000349[3]	
			3.2	0.0709	
			5.6	0.000893	
			10	0.2753	
			10	0.2723[4]	
24	13-03-12	11-04-12	0	0.0000620[3]	41
			1	0.000569[3]	2.0
			1.8	0.000980	280
			3.2	0.000475[3]	0.67
			5.6	0.0000972[3]	10.9
			10	0.000949	0.34
			10	0.0201[4]	7.4
72	15-03-12	11-04-12	0	n.d.	n.a.
			1	0.00136	4.9
			1.8	0.0000761[3]	22
			3.2	0.00296	4.2
			3.2[5]	0.00935	13
			5.6[6]	0.000447[3]	50
			5.6[7]	0.000425[3]	48
			5.6[8]	0.000175[3]	20
			10	0.0225	8.2
			10	0.00105[4]	0.39

1 Samples were stored in the freezer (≤ -15°C) until the day of analysis.
2 A water accommodated fraction (WAF) prepared at the loading rate
3 Obtained by extrapolation of the calibration curve
4 Without algae.
5 Sample taken from vessel 3.
6 Sample taken from vessel 1.
7 Sample taken from vessel 2.
8 Sample taken from vessel 3.
n.d. Not detected.
n.a. Not applicable.

Table 4 Concentrations of the test substance in test medium – second main study

Time of sampling [hours]	Date of sampling [dd-mm-yy]	Date of analysis [1] [dd-mm-yy]	Loading rate [2] [mg/l]	Concentration analysed [mg/l]	Relative to initial [%]
0	26-03-12	11-04-12	0	0.0000984[3]	
			1	0.0331	
			1.8	0.000440[3]	
			3.2	0.0516	
			5.6	0.0177	
			10	0.0152	
			10	0.0201[4]	
24	27-03-12	11-04-12	0	0.000134[3]	136
			1	0.00253	7.6
			1.8	0.0000943[3]	21
			3.2	0.00203	3.9
			5.6	0.000300[3]	1.7
			10	0.00117	7.7
			10	0.000570[3,4]	2.8
72	29-03-12	11-04-12	0	n.d.	n.a.
			1	0.00170	5.1
			1.8	0.000177[3]	40
			3.2	0.00219	4.3
			3.2[5]	0.000376[3]	0.73
			5.6	0.000330[3]	1.9
			10	0.000447[3]	2.9
			10	n.d. [4]	n.a.

1 Samples were stored in the freezer (≤ -15°C) until the day of analysis.
2 A water accommodated fraction (WAF) prepared at the loading rate.
3 Obtained by extrapolation of the calibration curve.
4 Without algae
5 Sample taken from vessel 3.
n.d. Not detected.
n.a. Not applicable.

APPENDIX 6 PROTOCOL

PROTOCOL

Study Title

FRESH WATER ALGAL GROWTH INHIBITION TEST WITH [redacted] (OIL FREE)

Author

Ing. M.H.J. Migchielsen

Test Facility

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Laboratory Project Identification

NOTOX Project 498471
NOTOX Substance 203662/A

Final Report

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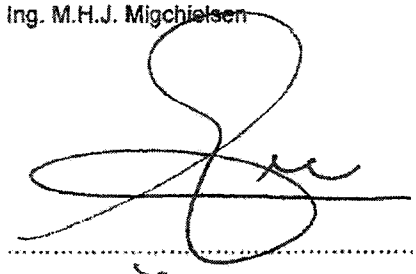
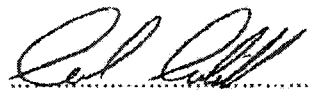
2. PROTOCOL APPROVAL

STUDY DIRECTOR: Ing. M.H.J. Migchielsen



date: 19 January 2012

HEAD OF QUALITY ASSURANCE: C.J. Mitchell B.Sc.



date: 20-Jan-2012

SPONSOR: [redacted]

date: 30-Jan-2012

3. INTRODUCTION

3.1. Preface

Sponsor	R.T. Vanderbilt Company, Inc. [redacted]
Study Monitor	Mr. R. Balcomb Director, Toxicology and Environmental Assessments Intertek Regulatory Services 1035 17th Street No.4 SANTA MONICA, CA 90403 USA
Test Facility	NOTOX B.V. Hambakenwetering 7 5231 DD „s-Hertogenbosch The Netherlands
Study Director	Ing. M.H.J. Migchielsen marcel.migchielsen@notox.nl
Technical Coordinator	R.W.A.M. Coolen
Principal Scientist	Dr. K.A. Oudhoff
Study Plan	Start week beginning : 20 February 2012 (week 08) Completed week beginning : 26 March 2012 (week 13) Proposed Reporting date : 06 May 2012

3.2. Aim of the study

The purpose of the study is to evaluate the test substance for its ability to generate toxic effects in *Pseudokirchneriella subcapitata* during an exposure period of at least 48 and at most 96 hours and, if possible, to determine the EC_{50} for both reduction of growth rate and inhibition of yield.

3.3. Guidelines

The study procedures described in this protocol are based on the Organization for Economic Co-operation and Development (OECD), OECD guidelines for Testing of Chemicals, guideline No. 201: "Freshwater Alga and Cyanobacteria, Growth Inhibition Test", Adopted March 23, 2006; Annex 5 corrected 28 July 2011.

In addition, the procedures are designed to meet the test methods prescribed by the following guidelines:

- Commission regulation (EC) No. 440/2008 of 30 May 2008, Part C: Methods for the determination of ecotoxicity, Publication No. L142, C3: "Algal Inhibition Test"; Amended by EC No. 761/2009 of 23 July 2009, Publication No. L220.
- ISO International Standard 8692: "Water quality - Freshwater algal growth inhibition test with unicellular green algae", Second edition, 01 October 2004.

And, if applicable, the following guidance document will be followed:

- Guidance document on aquatic toxicity testing of difficult substances and mixtures, OECD series on testing and assessment number 23, December 14, 2000.

3.4. Good Laboratory Practice

The study will be performed according to:

The Organization for Economic Cooperation and Development (OECD) Good Laboratory Practice Guidelines (1997).

Which essentially conform to:

The United States Food and Drug Administration Good Laboratory Practice Regulations.

The United States Environmental Protection Agency Good Laboratory Practice Regulations.

3.5. Quality Assurance

Study and/or process inspections will be performed by the NOTOX Quality Assurance Unit to assure the GLP compliance of this study. Facility inspections are also performed at regular intervals to assure the GLP compliance of general aspects.

The protocol will be inspected to confirm that it complies with GLP regulations. The report will be inspected to confirm that the methods and results accurately and completely reflect the raw data.

3.6. Storage and retention of records and materials

Records and materials pertaining to the study, including protocol, raw data, specimens (except specimens requiring refrigeration or freezing) and the final report, will be retained in the NOTOX archives for a period of at least 2 years after finalization of the report. After this period, the sponsor will be contacted to determine how the records and materials should be handled. NOTOX will retain information concerning decisions made.

Those specimens requiring refrigeration or freezing will be retained by NOTOX for as long as the quality of the specimens permits evaluation but no longer than three months after finalization of the report.

NOTOX will retain a test substance sample until the expiry date, but no longer than 10 years after finalization of the report. After this period the sample will be destroyed.

3.7. Definitions

Cell density is the number of cells per millilitre.

Growth rate is the increase in cell density per unit time. It is derived from the slope of the growth curve in a logarithmic plot. Following from the mathematical nature of exponential growth, the measure of the specific growth rate is preferable over biomass or yield. The E_RC_{50} is the concentration of test substance that results in a 50% reduction in growth rate relative to the control.

Yield is defined as the biomass at the end of the exposure period minus the biomass at the start of the exposure period. The E_yC_{50} is the concentration of test substance that results in a 50% inhibition of yield relative to the control.

No Observed Effect Concentration (NOEC) is the highest tested concentration at which the measured parameter(s) show(s) no significant effect on algal growth relative to control values.

If appropriate, additional definitions may be included in the report (e.g. definitions referring to poorly soluble substances).

4. MATERIALS AND METHODS

4.1. Test substance

The sponsor is responsible for Good Laboratory Practice (GLP) compliance for all test substance information unless determined by NOTOX. This will be specified in the GLP compliance statement in the report.

4.1.1. Test substance information

Identification	[redacted] (oil free)
Molecular formula	[redacted]
CAS Number	1159919-46-6
Description	Clear yellow viscous liquid (determined at NOTOX)
Batch	PB-39-131
Purity	UVCB
Test substance storage	At room temperature in the dark
Stability under storage conditions	Stable
Expiry date	01 December 2012 (allocated by NOTOX, 1 year after receipt of the test substance)

Cite publicly per PMN and TSCA Inventory list nomenclature as: Amines, bis (C11-14-branched and linear alkyl) tungstates.

4.1.2. Study specific test substance information

Hygroscopic	Not indicated
Volatile	Not indicated
Density	1.23 g/mL
Stability at higher temperatures	Not indicated
Stability in vehicle:	
• Water	Not indicated
• Dimethyl sulphoxide	Not indicated
• Ethanol	Not indicated
• Acetone	Not indicated
Solubility in vehicle:	
• Water	Insoluble
• Dimethyl sulphoxide	Soluble when hot
• Ethanol	Soluble
• Acetone	Soluble

4.1.3. Safety precautions and disposal category

Safety precautions	Gloves, goggles and face mask to ensure personnel health and safety
Disposal category	III

4.1.4. Reference substance

The results of the most recent reference test with potassium dichromate (Merck, Art. 4864) will be appended to the report. This reference test will have been performed a maximum of 3 months before or after the start of this project.

4.2. Test system

Species	*Pseudokirchneriella subcapitata*
Source	In-house laboratory culture.

Reason for selection	This system is an unicellular algal species sensitive to toxic substances in the aquatic ecosystem and has been selected as an internationally accepted species.

4.3. Fresh water algae culture

Stock culture	Algae stock cultures are started by inoculating growth medium with algal cells from a pure culture on agar. The suspensions are continuously aerated and exposed to light in a climate room at a temperature of 21-24°C.
Light intensity	60 to 120 µE/m^2/s when measured in the photosynthetically effective wavelength range of 400 to 700 nm.
Stock culture medium	M1; according to the NPR 6505, formulated using Milli-RO water and with the following composition:

$NaNO_3$	500	mg/l
$K_2HPO_4.3H_2O$	52	mg/l
$MgSO_4.7H_2O$	75	mg/l
Na_2CO_3 $10H_2O$	54	mg/l
$C_6H_8O_7.H_2O$	6	mg/l
NH_4NO_3	330	mg/l
$CaCl_2$ $2H_2O$	35	mg/l
$C_6H_5FeO_7.xH_2O$	6	mg/l
H_3BO_3	2.9	mg/l
$MnCl_2.4H_2O$	1.81	mg/l
$ZnCl_2$	0.11	mg/l
$CuSO_4$ $5H_2O$	0 08	mg/l
$(NH_4)_6Mo_7O_{24}.4H_2O$	0.018	mg/l

Pre-culture	2 to 4 days before the start of the test, cells from the algal stock culture are inoculated in culture medium at a cell density of 1 x 10^4 cells/ml. The pre-culture is maintained under the same conditions as used in the test. The cell density is measured immediately before use.
Pre-culture medium	M2; according to the OECD 201 Guideline, formulated using Milli-Q water preventing precipitation and with the following composition:

NH_4Cl	15	mg/l
$MgCl_2.6H_2O$	12	mg/l
$CaCl_2.2H_2O$	18	mg/l
$MgSO_4.7H_2O$	15	mg/l
KH_2PO_4	1.6	mg/l
$FeCl_3.6H_2O$	64	µg/l
$Na_2EDTA.2H_2O$	100	µg/l
H_3BO_3	185	µg/l
$MnCl_2.4H_2O$	415	µg/l
$ZnCl_2$	3	µg/l
$CoCl_2$ $6H_2O$	1.5	µg/l
$CuCl_2.2H_2O$	0 01	µg/l
$Na_2MoO_4.2H_2O$	7	µg/l
$NaHCO_3$	50	mg/l
Hardness (Ca+Mg)	0.24	mmol/l (24 mg $CaCO_3$/l)
pH	8 1 ± 0.2	

4.4. Preparation of stock and test solutions

The procedure for preparation of test solutions will be based on the available test substance information and/or on a pre-test.

The standard test procedures require generation of test solutions, which contain completely dissolved test substance concentrations or stable, and homogeneous mixtures or dispersions. The testing of concentrations that disturb the test system will be prevented or avoided, e.g. film of the test substance

on the water surface or extensive precipitation, flocculation, aggregation or deposition of the undissolved fraction of the test substance. The method of preparation of the test solutions will be based on data of the test substance supplied by the sponsor and/or on the results of a preliminary test (or specific tests with the test substance performed by NOTOX when the sponsor requests these). If applicable, the method of preparation will alternatively be based on the principles laid down in the OECD Guidance Document on Aquatic Toxicity Testing of Difficult Substances and Mixtures and referred to in the OECD Guidance Document On The Use Of The Harmonised System For The Classification Of Chemicals Which Are Hazardous For The Aquatic Environment, section 3.5: "Difficult to test substances".

The tests will be carried out without adjustment of the pH, except if pH values of test solutions are outside the optimal pH range for the species to be tested.

4.5. Range-finding test

A range-finding test will be performed to provide information about the range of concentrations to be used in the final test. Test procedure and conditions will be similar to those applied in the final test with the following exceptions:
Exponentially growing algal cultures will be exposed to a range of 0.1 to 100 mg/l increasing by a factor of 10 and to a control. If applicable a range will be tested up to and including the maximum solubility if this is below 100 mg/l. Standardly, one extra test vessel per concentration without algae will be used as background for the determination of the algal cell density at each time interval. Three replicates are tested per concentration and three replicates in the control group. pH will at least be measured in the control and the highest test concentration. At the end of the test algae will not be observed to verify a normal and healthy appearance. No sampling for determination of actual test concentrations will be performed.

Depending on the solubility of the test substance in test medium and, if appropriate the expected level of toxicity, the range of concentrations in the range-finding test may be different or include less concentrations. Alternatively, different methods of preparation may be combined in order to reach the expected water solubility (and dilutions thereof).

If no toxicity is expected, based on the characteristics of the test substance or other specific information, a limit test or alternatively a limit test combined with a range-finding test will be performed.

4.5.1. Limit test

The limit test will consist of a control and a concentration of 100 mg/l or, if applicable, a saturated solution whichever is lower. Test procedure and conditions will be similar to those applied in the final test except that 6 replicates will be used for both test groups. Samples for determination of actual exposure concentrations will be taken from the control and the test concentration at the start and at the end of the test. Optionally, samples can also be taken after 24 hours of exposure. No further testing will be required if no effects are observed and the validity criteria are met.

Depending on the solubility of the test substance in test medium different methods of preparation may be combined in order to reach the expected water solubility.

4.5.2. Combined limit/range-finding test

In a combined limit/range-finding test, 6 replicates of exponentially growing algae will be exposed to a control and a concentration of 100 mg/l or, if applicable, a saturated solution whichever is lower. Three replicates per concentration will be exposed to 0.1, 1.0 and 10 mg/l, or if applicable a range of dilutions containing 0.1, 1.0 and 10% of the saturated solution. pH will at least be measured in the control and the highest test concentration. Samples for determination of actual exposure concentrations will be taken from at least the control and the highest test concentration at the start and at the end of the test. Optionally, samples can also be taken after 24 hours of exposure. No further testing will be required if no effects are observed and the validity criteria are met. Observation of the algae to verify a normal and healthy appearance will only be performed in case of a limit test.

Depending on the solubility of the test substance in test medium and, if appropriate the expected level of toxicity, the range of concentrations in the range-finding test may be different or include less concentrations. Alternatively, different methods of preparation may be combined in order to reach the expected water solubility (and dilutions thereof).

4.6. Final test

4.6.1. Test concentrations

Number	At least 5 concentrations in a geometric series with a factor ≤ 3.2, except when the EC_{50} is expected to be greater than the maximum concentration to be tested. In that case a limit test can be performed.
Range	Preferably, the concentration range has to cover at least one concentration causing no-5% effects, one or more concentrations causing 5 to 75% effects and one concentration causing 75-100% effects with a standard maximum concentration of 100 mg/l or, if applicable, a saturated solution whichever is lower. The range may however include concentrations above 100 mg/l if this is relevant for the calculation of an EC_{50}-value.
Controls	Test medium without test substance or other additives or, if relevant, a control containing test medium with the additive used in the treatment of the stock solutions.
Replicates	3 replicates of each test concentration, 6 replicates of the controls, 1 replicate without algae at the highest test concentration, and, if relevant, 1 replicate of each test concentration without algae (turbidity control). If relevant, one or more extra replicates for sampling purposes.

4.6.2. Test procedure and conditions

Test duration	72 hours (standard) 48 hours for volatile substances
Test type	Static
Test vessels	100 ml, all-glass
Medium	M2
Cell concentration	An initial cell density of 1×10^4 cells/ml using an exponentially growing preculture.
Illumination	Continuously using TLD-lamps of the type „Cool White" of 30 Watt with a light intensity within the range of 60 to 120 $\mu E.m^{-2}.s^{-1}$
Temperature of medium	21-24 °C, constant within 2°C

pH	Between 6.0 and 9.0 Should not vary by more than 1.5 unit at the end of the test in any test solution. However, if the pH at the end of the test period had increased above 9.0 and/or varies by more than 1.5 unit and this increase is solely related to a relatively high rate of algal growth, this will be accepted.
Incubation	Capped vessels are distributed at random in the incubator and as such are daily repositioned. During incubation the algal cells are kept in suspension by continuous shaking, thereby improving gas exchange and reducing pH variation in the test solutions.

4.6.3. Sampling for analysis of test concentrations

Frequency	At the start and the end of the test. If the test concentrations are expected to be unstable, extra samples will be taken after 24 hours following exposure. Alternatively, sampling may be limited to those test solutions that are biologically relevant. If analytical results show that a concentration has decreased below the LOD/LOQ before the end of the test period, no further sampling is needed at that concentration.
Concentrations (standard[1])	Samples will be taken from at least three concentrations, i.e. the lowest, a middle and the highest, and the control(s) for analysis. At the start of the test care will be taken not to include any floating layer, test substance film or undissolved material in separate vessels. At the end of the test samples will be taken from the approximate centre of the pooled solutions of the vessels containing the algal suspensions at each concentration.
Number of samples	Sampling will consist of singular samples per treatment. Should the analytical validation require duplicate or multiple samples per treatment, this will be followed without prior notification. In case undissolved particles were removed from the test solutions before the start of the test, this residue will be retained for possible analysis.
Volume	Standardly, volumes of 2 ml will be taken, but depending on the limit of detection of the analytical method used in relation to the test concentrations the volume may differ.
Storage	If stability of test concentrations under deep-freeze conditions is ensured, the samples will be stored in a deep-freezer until analysis. Optionally, samples can be stored under different conditions (e.g. room temp. or in refrigerator) if stability under these conditions is ensured.

[1] The standard frequency of sampling is only applicable provided that test solutions are diluted using **one** stock and test concentrations should be **above** 1 mg/l. Sampling will include **all** test solutions if the previous mentioned conditions are not met or if the Study Director decides this is essential for other reasons. Alternatively, sampling may be limited to those test solutions that are biologically relevant.

Extra samples	In case singular samples are taken, which are known to be stable under the storage conditions, extra samples will be taken and stored for possible analysis until delivery of the final report with a maximum of three months.
Abiotic control	Compliance with the criteria for maintenance of actual concentrations will be demonstrated by running a test vessel without algae at the highest substance concentration and samples for analysis will be taken at the start and the end of the test period.
Analyses	Preferably, the entire volume of each sample used for analysis will be taken for further dilution or pre-treatment. The analytical method used will be based on the results of a separate project for the development and validation of the analytical method. If study specific adjustments of the analytical method or sample pre-treatment procedures are necessary, these will be developed and tested before the performance of the final test. Detailed specification of these additional analytical procedures will be put down in a protocol amendment (see also 'Additional procedures').

4.6.4. Measurements and recording

pH	At the beginning and at the end of the test in at least one vessel per concentration. The pH of the solution should preferably not deviate by more than 1.5 units during the test.
Temperature of medium	Continuously in a temperature control vessel.
Appearance of the cells	At the end of the final test microscopic observation will be performed on at least one of the test concentrations with sufficient algal growth to verify a normal and healthy appearance of the inoculum culture and to observe any abnormal appearance of the algae.
Cell densities:	
Clear solutions	At the beginning of the test, cell density is based on counting by microscope using a counting chamber. Thereafter cell densities are determined daily by spectrophotometric measurement of samples at 720 nm using a spectrophotometer with immersion probe (path length =20 mm) or with cuvettes (path length = 10 mm). Algal medium will be used as a blank.
Turbid solutions	If the test solutions are slightly turbid one extra test vessel per concentration without algae will be used as background for the determination of the algal cell density at each time interval. If the test solutions are so turbid that they disturb the spectrophotometric measurements substantially, the algal densities will be recorded by direct counting using a microscope.

4.7. Specific items for Study Director approval in study files

The following items will be approved in the study files by the Study Director:

- Choice of range-finding test, combined limit/range-finding test or limit test
- Concentrations to be tested
- Procedure(s) for preparation of test solutions
- Sampling and analysis:
  - Number and volume of samples to be taken
  - Treatment of samples
  - Samples to be analysed

4.8. Electronic data capture

Observations/measurements in the study will be recorded electronically using the following programme(s):
- Shimadzu Spectrophotometer UV-1800 including UVProbe 2.33 software (Shimadzu, Kyoto, Japan): Algal cell density.
- REES Centron Environmental Monitoring system version SQL 2.0 (REES Scientific, Trenton, NJ, USA): Temperature.

System control, data acquisition and data processing for analytical chemistry will be performed using one or more of the following programmes:

- Empower version 7.00 (Waters, Milford, MA, USA)
- Enhanced Chemstation version D.00.01.27 (Agilent Technologies, Wilmington, DE, USA)
- MassLynx version 4.1 (Waters, Milford, MA, USA)
- Xcalibur version 2.0 (Thermo, San Jose, CA, USA)
- ICP-MS Chemstation version B.03.04 (Agilent Technologies, Tokyo, Japan)
- ICP-MS Chromatographic software version C.01.00 (Agilent Technologies, Tokyo, Japan)

The actual programme(s) will be approved in the raw data and reported.

Any upgrades will be approved by the Study Director (or Principal Scientist/Investigator) in the study files.

4.9. Interpretation

4.9.1. Acceptability of the test

1. The cell density in the control cultures should have increased by a factor of at least 16 within the exposure period.
2. The mean coefficient of variation for section-by-section specific growth rates in the control cultures must not exceed 35%.
3. The coefficient of variation of average specific growth rates during the whole test period in replicate control cultures must not exceed 7%.

If (one of) the acceptability criteria are not met and the Study Director decides that this has a critical effect on the study, the test will be rejected and repeated.

4.9.2. Additional procedures

Additional or alternative procedures will be required for the testing of:

1. Volatile substances;
2. Very toxic or low soluble substances (test concentrations < 1 mg/l);
3. Hydrolytically unstable or photosensitive substances;
4. Dye stuffs;
5. pH affecting substances;
6. Substances that are not stable under deep-freeze conditions.

These additional procedures may require the amending of:

1. Preparation of test solutions;
2. The frequency of sampling and analysis for the determination of actual test concentrations;
3. Extension of the analytical program with respect to sample treatment and the sensitivity of the analytical method;
4. In case of a dye stuff: additional testing to compare the effect on algal growth induced by the color of the test solutions with the results of the toxicity test.
5. If applicable, additional testing with pH adjustment.

The additional procedures are no part of the standard test procedures and will be applied only after emission of an authorised protocol amendment. In such a case the amended procedures will be effective only after NOTOX has received any kind of authorisation from the study monitor.

4.9.3. Data handling

Defining exposure concentration

1. The results will be based on the nominal or initial (if not in agreement with nominal) test substance concentrations if the analytical program has confirmed that the measured test substance concentrations remained within 20% of the nominal or initial concentrations.
2. If the deviation of the exposure concentrations of the test substance is greater than ± 20% of the nominal or initial concentrations, the results will be expressed in terms of average exposure concentrations. Where measured data are available for the start and end of the test, these concentrations are geometric means calculated from the concentrations measured at the start and end of the test. In case that additional analysis is performed after 24 hours, a time weighed average concentration is calculated. Where at the end of the test measured concentrations are below the analytical detection limit, such concentrations shall be considered to be half that detection limit.

Calibration curve

At the start of the algal test, a calibration curve will be made using a minimum of six dilutions of one or two of the pre-cultures. The software of the Shimadzu Spectrophotometer will be used to plot cell density against extinction. The software automatically calculates the cell densities based on this curve for the spectrophotometric measurements at the various points in time during the test period.

Comparison of average growth rates

The average specific growth rate for a specific period is calculated as the logarithmic increase in the biomass from the equation for each single vessel of controls and treatments:

$$\mu_{i-j} = \frac{\ln X_j - \ln X_i}{t_j - t_i} (\text{day}^{-1})$$

Where: μ_{i-j} = the average specific growth rate from time i to j
X_i = the biomass at time i
X_j = the biomass at time j

The average growth rate at each test substance concentration is then compared with the control value and the percentage reduction in growth rate is calculated:

$$\%I_r = \frac{\mu_C - \mu_T}{\mu_C} \times 100$$

Where: $\%I_r$ = percent inhibition in average specific growth rate
μ_C = mean value for average specific growth rate in the control group
μ_T = average specific growth rate for the treatment replicate

Yield

The percent inhibition in yield (biomass at the end of the exposure period minus the biomass at the start of the exposure period) is calculated for each treatment replicate as follows:

$$\%I_y = \frac{Y_C - Y_T}{Y_C} \times 100$$

Where: $\%I_y$ = percent inhibition of yield
Y_C = mean value for yield in the control group
Y_T = value for yield for the treatment replicate

Determination of the effect parameters (NOEC, EC_{50}, EC_{10})

The percentages of growth rate reduction and/or the percentages of yield inhibition will be set out against the logarithms of the corresponding concentrations of the test substance. If a test concentration related effect is present, calculation of the EC-values will be based on the respective curves corresponding to the various observation times using regression analysis. If possible, also the EC_{10} will be determined to meet the recommendations as put down in "A Review of Statistical Data Analysis and Experimental Design in OECD Aquatic Toxicology Test Guidelines" by S. Pack, August 1993.

If required, a NOEC will be determined. The NOEC will be based on statistical analysis of the data. Data obtained for the test concentrations will be compared with those obtained in the solvent or negative control using TOXSTAT Release 3.5, 1996, D.D. Gulley, A.M. Boelter, H.L. Bergman.

Optionally, other statistical analyses may be performed if appropriate.

5. DISTRIBUTION

Original:	Study Director
1 Copy:	Technical Coordinator
1 Copy:	Analytical Chemistry
1 Copy:	QAU/Management
1 Copy:	Sponsor

PROTOCOL AMENDMENT NO : 1

Study Title	FRESH WATER ALGAL GROWTH INHIBITION TEST WITH VANLUBE®W-324 (oil free)
Sponsor	[redacted]
Study Monitor	Mr. R. Balcomb Director, Toxicology and Environmental Assessments Intertek Regulatory Services 1035 17th Street No.4 SANTA MONICA, CA 90403 USA
NOTOX Substance	203662/A
NOTOX Project	498471

AMENDMENT DESCRIPTION

1. Page 4, Preface, Principal Scientist:

   Principal scientist will be changed to E. Baltussen, PhD.

REASONS FOR AMENDMENT

1. Formal replacement as K.A. Oudhoff, PhD is no longer working for NOTOX B.V.

APPROVAL
Study director

.....
M.H.J. Migchielsen, Bachelor

17 August 2012
.....
date:

[redacted]

351920

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RECEIVED
OPPT CBIC

February 8, 2012

TSCA Confidential Business Information Center (7407M)
EPA East - Room 6428 Attn: Section 8(e)
U.S. Environmental Protection Agency
1200 Pennsylvania Avenue, N.W.
Washington, DC 20460-0001

Re: Submission Pursuant to Section 8(e) of the Toxic Substances Control Act ("TSCA"): Acute Toxicity Study for Amines, bis (C11-14-branched and linear alkyl), tungstates

Dear Sir or Madam:

On January 9, 2012, the [redacted] ("the Company") received a Final Draft of an acute toxicity study ("Final Study") for one of the substances used in [redacted] ("The Product").[1] As described in the original pre-manufacture notice, the specific substance tested ("Tested Substance") is identified as "Amines, bis (C11-14-branched and linear alkyl), tungstates"[2] and its CAS registry number is 1159919-46-6. The testing laboratory, NOTOX B.V. ("NOTOX"), assessed the Tested Substance for acute toxicity to daphnia. The test was conducted in a static system over 48 hours in accordance with OECD guideline No. 202, 2004. In addition, the procedures were designed to meet the test methods of the Commission Regulation (EC) No 440/2008, Part C.2, 2008, the ISO International Standard 6341, 1996 and the OECD series on testing and assessment number 23, 2000.

The findings of the Final Study are consistent with the findings reported in the Draft Study, previously submitted to EPA on June 25, 2012. The Final Study concluded that the Tested Substance had a 48 hour EC50 of 19 µg/l (95% confidence interval between 14 and 30 µg/l).

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[1] Portions of this letter claimed as confidential are bracketed and highlighted in **bold**.

[2] The Study itself, conducted in Europe, includes references to an alternative descriptive formula prepared for a confidential submission to the European Chemicals Agency under REACH. *See* Study at 6. Because this alternative descriptive formula was not included in the pre-manufacture notice ("PMN") or on the TSCA inventory, and because the alternative nomenclature would provide competitors with information relevant to the manufacturing process for the substance itself, the Company is claiming it as confidential business information ("CBI"), and has annotated the public version of the study to reference the nonconfidential US nomenclature.

Company Sanitized

Please note that the Company has not made a determination as to whether a substantial risk of injury to health or the environment is actually presented by these findings. Recognizing, however, that EPA could interpret such information as constituting a substantial risk when considered with other studies submitted to the Agency, the Company is submitting the study under TSCA §8(e) out of an abundance of caution.

Enclosed are confidential and redacted public versions of the Study, this cover letter, and a detailed justification for confidential treatment of the Company's identifying information, the trade name for the Product containing the Tested Substance, and the alternative descriptive formula.

If you have any questions or need more information, please contact me at 203-295-2143 Ext 264.

Sincerely yours,

[redacted]

Enclosures

cc: [redacted]

Attachment 1: Substantiation for Confidentiality Claims

Substantiation Questions

1. **Is your company asserting this confidential business information (CBI) claim on its own behalf? If the answer is no, please provide company name, address and telephone number of entity asserting claim.**

   Company asserts this CBI claim on its own behalf.

2. **For what period do you assert your claim(s) of confidentiality? If the claim is to extend until a certain event or point in time, please indicate that event or time period. Explain why such information should remain confidential until such point.**

   The Company asserts an indefinite claim of confidentiality with respect to three categories of information: a) The Company's name and address; b) the Trade Name and identity of a proprietary product containing the Tested Substance, and c) an alternative descriptive formula for the Tested Substance prepared by NOTOX for a confidential submission to the European Chemicals Agency. Each is discussed in turn.

   a. **The Company's name, address, and other identifying information.**

      The cover letter and page 6, 29, and 30 of the Study reference the Company's name and address. The Company claims this information as CBI for an indefinite period.

      Disclosure of Company information and the Product Trade Name would disclose confidential business information relating to the Company's extensive research, development, and commercialization efforts to evaluate, identify, and select specific substances with exceptional performance characteristics in competitive markets. Disclosure would also provide competitors with sensitive and confidential information on specific details of the proprietary ingredients used in specific Company products. Disclosure of the name of the Company submitting this test would also disclose confidential business information regarding business relationships the Company has established with specific third-party testing laboratories.

   b. **The Trade Name and identity of a Proprietary Product.**

      The cover letter and each page of the Study reference the Trade Name and identity of a Product containing the Tested Substance. The Company claims this information to be CBI for an indefinite period.

      As with the Company information, disclosure of the Product Trade Name would release confidential business information relating to the Company's extensive research, development, and commercialization efforts to evaluate, identify, and select specific substances with exceptional performance characteristics in competitive markets. Disclosure of Company and Product Trade Names would

also provide competitors with sensitive and confidential information on specific details of the proprietary ingredients used in specific Company products. Finally, disclosing the Product Name would, in turn, disclose the identity of the Company that commissioned this test, thus disclosing CBI regarding business relationships the Company has established with specific third-party testing laboratories.

c. **Alternative Descriptive Formula.**

Pages 7 and 31 of the Study reference an alternative descriptive formula for the Tested Substance prepared by NOTOX as part of a confidential submission to the European Chemicals Agency under REACH. The alternative descriptive formula refers to the same Tested Substance identified in the Company's PMN and the TSCA Inventory as "Amines, bis(C11-14-branched and linear alkyl), tungstates," CAS No. 1159919-46-6. Unlike the descriptive formula contained in the PMN and on the TSCA Inventory, however, the confidential alternative descriptive formula would provide competitors with more detailed information that could reveal elements of the manufacturing process for the substance itself. As such, the Company is claiming the alternative descriptive formula to be confidential business information and has annotated the public version of the study to include reference to the nonconfidential US nomenclature.

3. **Has the information that you are claiming as CBI been disclosed to any other Governmental Agency, or to this Agency at any other time?**

The trade name was disclosed on the PMN, but was marked as CBI. The trade name and alternative descriptive formula were disclosed in a submission to the Environmental Chemicals Agency pursuant to Article 26 of REACH. This submission, however, was submitted as confidential and is not available to the public.

4. **Briefly describe any physical or procedural restrictions within your company relating to the use and storage of the information you are claiming CBI.**

CBI is kept secure in locked file cabinets and its distribution is restricted to company personnel on an as need to know basis. All computer networks containing information are secured and protected by firewalls.

5. **If anyone outside your company has access to any of the information claimed CBI, are they restricted by confidentiality agreement(s)? If so, explain the content of the agreement(s).**

While the Tested Substance is listed on the Toxic Substances Inventory, its use in the Product is CBI. Such information is shared with vendors on a need to know basis, and only under stipulations of confidentiality preventing the distribution of such information.

6. **Does the information claimed as CBI appear or is it referred to in any of the following:**

   a. **Advertising or promotional material for the chemical substance or the resulting end product;**

      No. These materials would not link the Tested Substance to the Company or a specific Company Product by CAS Number or substance composition.

   b. **Material safety data sheets or other similar materials (such as technical data sheets) for the substance or resulting end product (include copies of this information as it appears when accompanying the substance and/or product at the time of transfer or sale);**

      No. These materials would not link the Tested Substance to the Company or a specific Company Product by CAS number or substance composition.

   c. **Professional or trade publications; or**

      No. These materials would not link the Tested Substance to the Company or a specific Company Product by CAS Number or substance composition.

   d. **Any other media or publications available to the public or to your competitors.**

      No. These materials would not link the Tested Substance to the Company or a specific Company Product by CAS Number or substance composition.

7. **Has EPA, another federal agency, or court made any confidentiality determination regarding information associated with this substance? If so, provide copies of such determinations.**

   No.

8. **Describe the substantial harmful effects that would result to your competitive position if the CBI is made available to the public.**

   As noted above, the Company is not seeking to limit the public availability of the health and safety data in the study or the CAS number and name of the specific substance tested. Rather, the CBI claims extend to the identifying information for the Company itself and the Proprietary Product(s) in which the Tested Substance is used. The Company expends considerable resources on research and development to identify which substances provide the highest level of performance and value for customers, and this information would have significant value to competitors seeking to compete in similar markets. While the CAS Number and structural identity of the Tested Substance is publicly available, releasing information on its use in specific Company products would undermine the Company's competitive advantage by implicitly disclosing proprietary information on the value and utility of the substance for specific market uses. The

alternative descriptive formula, not required for the domestic registration, would provide competitors with additional information governing the manufacturing process for the substance.

9. **Has the substance been patented in the U.S. or elsewhere? Is a patent for the substance currently pending?**

Composition of matter patent. Patent filed and granted in the US. Patents filed and granted in India, Japan, China and Germany. These documents do not disclose the information claimed as CBI in this filing.

10. **Is this substance/product commercially available and if so, for how long has it been available on the commercial market?**

Yes. The Tested Substance has been commercially available since May 2009.

If on the commercial market, are your competitors aware that the substance is commercially available in the U.S.?

The MSDS states that the product contains "amines bis(C11-C14 alkyl) tungstates," but does not link the product to a specific CAS No. or disclose the specific product composition beyond a range.

a. **If not already commercially available, describe what stage of research and development (R&D) the substance is in, and estimate how soon a market will be established.**

[redacted]

b. **What is the substance used for and what type of product(s) does it appear in?**

[redacted]

11. **Describe whether a competitor could employ reverse engineering to identically recreate the substance.**

The Company does not oppose disclosing the identity of the substance itself, and it has not sought to claim the CAS number or the name as submitted in the PMN and as listed on the TSCA Inventory. Rather, the Company is concerned that disclosing the identity of the Manufacturer, the Trade Name of the Product in which it is used, and the alternative descriptive formula would allow a competitor to deduce confidential properties and commercial values of the Tested Substance. Moreover, the alternative descriptive formula would assist a competitor in determining the manufacturing process for the Tested Substance.

12. **Do you assert that disclosure of this information you are claiming CBI would reveal:**

a. **Confidential processes used in manufacturing the substance;**

Disclosure of the alternative descriptive formula would compromise confidential information regarding the manufacturing process for the substance.

b. **If a mixture, the actual portions of the substance in the mixture; or**

As stated on MSDS petroleum process oil, <3.0%, DMSO extractable material 64742-52-5 40 – 70% amines bis(C11-C14 alkyl) tungstates 30 – 60%.

c. **Information unrelated to the effects of the substance on human health or the environment?**

The Company does not oppose disclosing the identity of the substance itself, and it has not sought to claim the CAS number or substance name, as filed in the PMN, as CBI. Nor does the Company seek to restrict public access to the Study's findings on the potential effects of the Tested Substance on human health or the environment. Rather, the Company is concerned that disclosing the identity of the Manufacturer, the Trade Name of a product in which the Tested Substance is used, and the alternative descriptive formula would reveal sensitive market and economic information on the value of specific substances to specific market uses, its presence in specific proprietary Company products, and information regarding the manufacturing process.

13. **Provide the Chemical Abstract Service Registry Number for the product, if known.**

1159919-46-6.

14. **Is the substance or any information claimed CBI the subject of FIFRA regulation or reporting? If so, explain.**

No.

FINAL REPORT

Study Title

ACUTE TOXICITY STUDY IN *DAPHNIA MAGNA* WITH [redacted] (OIL FREE)

(STATIC)

Author

Ing. M.H.J. Migchielsen

Test Facility

NOTOX B.V.
Hambakenwetering 7
5231 DD 's-Hertogenbosch
The Netherlands

Laboratory Project Identification

NOTOX Project 498470
NOTOX Substance 203662/A

1. CONTENTS

2. STATEMENT OF GLP COMPLIANCE

NOTOX B.V., 's-Hertogenbosch, The Netherlands

The study described in this report has been correctly reported and was conducted in compliance with:

The Organization for Economic Co-operation and Development (OECD) Principles of Good Laboratory Practice (GLP) (as revised in 1997) ENV/MC/CHEM (98) 17.

The sponsor is responsible for Good Laboratory Practice (GLP) compliance for all test substance information unless determined by NOTOX.

NOTOX B.V.

Ing. M.H.J. Migchielsen
Study Director

Date: 23 August 2012

Ing. E.J. van de Waart, M.Sc.
Head of In Vitro & Environmental Toxicology

i.a. i.A.T.M. Meerts
Section Head

Date: 22 August 2012

3. QUALITY ASSURANCE STATEMENT

NOTOX B.V., 's-Hertogenbosch, The Netherlands

This report was inspected by the NOTOX Quality Assurance Unit to confirm that the methods and results accurately and completely reflect the raw data.

During the on-site process inspections, procedures applicable to this type of study were inspected.

The dates of Quality Assurance inspections are given below.

Type of Inspections	Phase/Process	Start Inspection date	End Inspection date	Reporting date
Study	Protocol	20-Jan-12	20-Jan-12	20-Jan-12
	Report	03-May-12	03-May-12	03-May-12
	Protocol Amendment 1	17-Aug-12	17-Aug-12	17-Aug-12
Process	**Environmental Toxicology** Test Substance Handling Exposure Observations/Measurements	30-Jan-12	03-Feb-12	03-Feb-12
	Analytical and physical chemistry Test Substance Handling Observations/Measurements	06-Feb-12	13-Feb-12	16-Feb-12

NOTOX B.V.

C.J. Mitchell B.Sc.
Head of Quality Assurance

Date: 22-Aug-2012.

4. SUMMARY

Acute Toxicity Study in *Daphnia magna* with [redacted] (oil free).

The study procedures described in this report were based on the OECD guideline No. 202, 2004. In addition, the procedures were designed to meet the test methods of the Commission Regulation (EC) No 440/2008, Part C.2, 2008, the ISO International Standard 6341, 1996 and the OECD series on testing and assessment number 23, 2000.

The batch of [redacted] (oil free) tested was a UVCB substance. The material was not completely soluble in the test medium at the initial loading rates prepared (indicated as "insoluble in cold water" on MSDS).

Preparation of test solutions started with individually prepared loading rates. Exact amounts of the viscous liquid were weighed and placed on cover slips. The cover slips were then transferred into measuring flasks that contained pre-heated (~35-40°C) test medium. Subsequently, a three-day magnetic stirring period was applied to ensure reaching maximum dissolution in test medium at the various loading rates. The resulting dispersions were left to settle for approximately 2 hours were after the Water Accommodated Fractions (WAFs) were collected and used for testing. The final test solutions were all clear and colourless.

A final test was performed based on the result obtained in a preceding combined limit/range-finding test. Twenty daphnia per test group (5 per vessel, 4 vessels) were exposed to a control and to WAFs prepared at loading rates of 0.46, 1.0, 2.2, 4.6 and 10 mg/l. The total test period was 48 hours and a static test system was applied. Samples for analyses of actual exposure concentrations were taken at the start and the end of the test period.

Analyses were only considered necessary for the WAFs essential for determination of the toxicity parameters, i.e. the WAFs prepared at 4.6 and 10 mg/l. Analyses showed that the measured concentrations were 8.7 µg/l and 105 µg/l, respectively. The measured concentrations decreased to 1.8 and 17.5 µg/l during the test period in the WAFs of 4.6 and 10 µg/l, respectively. The observed decrease was likely related to the very low solubility (indicated as "insoluble" by the sponsor).The average exposure concentrations for the WAFs prepared at 4.6 and 10 mg/l were respectively 4.0 and 43 µg/l.

The study met the acceptability criteria prescribed by the protocol and was considered valid.

[redacted] (oil free) did not induce acute immobilisation of *Daphnia magna* exposed to a WAF prepared at a loading rate of 4.6 mg/l after 48 hours of exposure. Analyses showed that this corresponded to an average exposure concentration of 4.0 µg/l (NOEC).

The 48h-EC_{50} was between concentrations present in WAFs prepared at loading rates of 4.6 and 10 mg/l. The 48h-EC_{50} based on average exposure concentrations was 19 µg/l (95% confidence interval between 14 and 30 µg/l).

5. INTRODUCTION

5.1. Preface

Sponsor	[redacted]
Study Monitor	Mr. R. Balcomb Director, Toxicology and Environmental Assessments Intertek Regulatory Services 1035 17th Street No.4 SANTA MONICA, CA 90403 USA
Test Facility	NOTOX B.V. Hambakenwetering 7 5231 DD 's-Hertogenbosch The Netherlands
Study Director	Ing. M.H.J. Migchielsen
Principal Scientist	E. Baltussen, PhD
Study Plan	Start : 06 February 2012 Completion : 14 March 2012

5.2. Aim of the study

The purpose of the study was to evaluate the test substance for its ability to generate acute toxic effects on the mobility of *Daphnia magna* during an exposure period of 48 hours and, if possible, to determine the EC_{50} at 24 and 48 hours of exposure.

5.3. Guidelines

The study procedures described in this report were based on the Organization for Economic Co-operation and Development (OECD), OECD guidelines for Testing of Chemicals, guideline No. 202: "*Daphnia sp.*, Acute Immobilisation Test", Adopted April 13, 2004.

In addition, the procedures were designed to meet the test methods of the following guidelines \and guidance document:

- Commission Regulation (EC) No 440/2008 of 30 May 2008, Part C: Methods for the determination of ecotoxicity, Publication No. L142, C.2. "Daphnia Sp. Acute Immobilisation Test".
- ISO International Standard 6341: "Water quality - Determination of the inhibition of the mobility of *Daphnia magna* Straus - Acute toxicity test, Third edition, 1996-04-01.
- Guidance document on aquatic toxicity testing of difficult substances and mixtures, OECD series on testing and assessment number 23, December 14, 2000.

5.4. Storage and retention of records and materials

Records and materials pertaining to the study including protocol, raw data, specimens (except specimens requiring refrigeration or freezing) and the final report are retained in the NOTOX archives for a period of at least 2 years after finalization of the report. After this period, the sponsor will be contacted to determine how the records and materials should be handled. NOTOX will retain information concerning decisions made.

Those specimens requiring refrigeration or freezing will be retained by NOTOX for as long as the quality of the specimens permits evaluation but no longer than three months after finalization of the

report.

NOTOX will retain a test substance sample until the expiry date, but no longer than 10 years after finalization of the report. After this period the sample will be destroyed.

5.5. Definitions

Immobile are those animals not able to swim within 15 seconds after gentle agitation of the test vessel.

The **EC_{50}** is the concentration of test substance estimated to immobilise 50% of the daphnids after a defined period of exposure.

No Observed Effect Concentration (NOEC) is the highest concentration tested at which no effect (i.e. immobilisation) is recorded.

6. MATERIALS AND METHODS

6.1. Test Substance

6.1.1. Test substance information

Cite publicly per PMN and TSCA Inventory list nomenclature as: Amines, bis (C11-14-branched and linear alkyl) tungstates.

Identification	[redacted] (oil free)
Molecular formula	[redacted]
CAS Number	1159919-46-6
Description	Clear yellow viscous liquid (determined at NOTOX)
Batch	PB-39-131
Purity	UVCB
Test substance storage	At room temperature in the dark
Stability under storage conditions	Stable
Expiry date	01 December 2012 (allocated by NOTOX, 1 year after receipt of the test substance)

6.1.2. Study specific test substance information

Volatile	Not indicated
Stability at higher temperatures	Not indicated
Stability in water	Not indicated
Solubility in water	Insoluble

6.1.3. Reference substance

This report includes the results of the most recent reference test with potassium dichromate (APPENDIX 2).

6.2. Test system

Species	*Daphnia magna* (Crustacea, Cladocera) (Straus, 1820), at least third generation, obtained by acyclical parthenogenesis under specified breeding conditions.
Source	In-house laboratory culture with a known history.
Reason for selection	This system has been selected as an internationally accepted invertebrate species.
Validity of batch	Daphnids originated from a healthy stock, 2nd to 5th brood,

	showing no signs of stress such as mortality >20%, presence of males, ephippia or discoloured animals and there was no delay in the production of the first brood.
Characteristics	For the test selection of young daphnids with an age of < 24 hours, from parental daphnids of more than two weeks old.

6.3. Breeding

Start of each batch	With newborn daphnids, i.e. less than 3 days old, by placing about 250 of them into 5 litres of medium in an all-glass culture vessel.
Maximum age of the cultures	4 weeks
Renewal of the cultures	After 7 days of cultivation half of the medium twice a week.
Temperature of medium	18-22°C
Feeding	Daily, a suspension of fresh water algae.
Medium	M7, as prescribed by Dr. Elendt-Schneider (Elendt, B.-P., 1990: Selenium deficiency in Crustacea. An ultrastructural approach to antennal damage in *Daphnia magna* Straus. Protoplasma 154, 25-33).

Composition of medium M7

Adjusted ISO medium. the following chemicals (analytical grade) are dissolved in tap water purified by Reverse Osmosis (RO-water, GEON Waterbehandeling, Berkel-Enschot, The Netherlands)

Macro salts:	$CaCl_2.2H_2O$	211 5	mg/l
	$MgSO_4\ 7H_2O$	88.8	mg/l
	$NaHCO_3$	46.7	mg/l
	KCl	4.2	mg/l

Medium M7 trace elements, macro nutrients and vitamins are added to freshly prepared ISO medium to reach the following concentrations.

Trace elements	B	0.125	mg/l
	Fe	0 05	mg/l
	Mn	0.025	mg/l
	Li, Rb and Sr	0 0125	mg/l
	Mo	0.0063	mg/l
	Br	0.0025	mg/l
	Cu	0.0016	mg/l
	Zn	0.0063	mg/l
	Co and I	0 0025	mg/l
	Se	0.0010	mg/l
	V	0 0003	mg/l
	$Na_2EDTA.2H_2O$	2.5	mg/l
Macro nutrients	$Na_2SiO_3.\ 9H_2O$	10.0	mg/l
	$NaNO_3$	0 27	mg/l
	KH_2PO_4	0.14	mg/l
	K_2HPO_4	0 18	mg/l
Vitamins	Thiamine	75.0	µg/l
	B_{12}	1.0	µg/l
	Biotin	0 75	µg/l

The hardness: 180 mg/l expressed as $CaCO_3$ and the pH 7.7 ± 0.3.

6.4. Preparation of test solutions

The standard test procedures required generation of test solutions, which should contain completely dissolved test substance concentrations or stable and homogeneous mixtures or dispersions. The testing of concentrations that disturb the test system should be prevented (e.g. film of the test substance on the water surface).

The batch of [redacted] (oil free) tested was a UVCB substance. The material was not completely soluble in the test medium at the initial loading rates prepared (indicated as "insoluble in cold water" on MSDS).

Preparation of test solutions started with individually prepared loading rates. Exact amounts of the viscous liquid were weighed and placed on cover slips. The cover slips were then transferred into measuring flasks that contained pre-heated (~35-40°C) test medium. Subsequently, a three-day magnetic stirring period was applied to ensure reaching maximum dissolution in test medium at the various loading rates. The resulting dispersions were left to settle for approximately 2 hours were after the Water Accommodated Fractions (WAFs) were collected and used for testing. The final test solutions were all clear and colourless.

6.5. Combined limit/range-finding test

The project started with a combined limit/range-finding test. Twenty daphnids per concentration (four replicates, 5 daphnids per vessel) were exposed to a control and a WAF prepared at a loading rate of 100 mg/l. Test procedure and conditions were similar to those applied in the final test with the following exceptions:

- Ten daphnids per concentration (in duplicate, 5 per vessel) were exposed to loading rates of 1.0 and 10 mg/l in the combined range-finding test.
- Dissolved oxygen concentrations and pH were only measured in the control and the highest test concentration.

6.6. Final test

6.6.1. Test concentrations

[redacted] (oil free)	WAFs prepared at loading rates of 0.46, 1.0, 2.2, 4.6 and 10 mg/l.
Controls	Test medium without test substance or other additives.

6.6.2. Test procedure and conditions

Test duration	48 hours
Test type	Static
Test vessels	100 ml, all-glass
Medium	Adjusted ISO medium
Number of daphnids	20 per concentration
Loading	5 per vessel containing 80 ml of test solution
Light	16 hours photoperiod daily
Feeding	No feeding
Aeration	No aeration of the test solutions.

Introduction of daphnids	Within half an hour after preparation of the test solutions.

6.6.3. Sampling for analysis of test concentrations

During the final test singular samples for possible analysis were taken from all test concentrations and the control according to the schedule below. The method of analysis is described in the appended Analytical Report (APPENDIX 3).

Frequency	at t=0 h and t=48 h
Volume	1.0 ml from the approximate centre of the test vessels
Storage	Samples were stored in a freezer until analysis.

At the end of the exposure period, the replicates were pooled at each concentration before sampling.

Additionally, reserve samples of 1.0 ml were taken for possible analysis. If not used, these samples were stored in a freezer for a maximum of three months after delivery of the draft report, pending on the decision of the sponsor for additional analysis.

6.6.4. Measurements and recordings

Immobility (including mortality)	At 24 hours and at 48 hours.
pH and dissolved oxygen	At the beginning and at the end of the test, for all concentrations and the control.
Temperature of medium	Continuously in a temperature control vessel, beginning at the start of the test.

6.7. Electronic data capture

Observations/measurements in the study were recorded electronically using the following programme(s):
- REES Centron Environmental Monitoring system version SQL 2.0 (REES Scientific, Trenton, NJ, USA): Temperature.

6.8. Interpretation

6.8.1. Data handling

Determination of the average exposure concentrations:

The average exposure concentrations were calculated as $\sqrt{C_{t=0} \times C_{t=48}}$, being the geometric means of the concentrations of [redacted] (oil free) measured in the samples taken at the start ($C_{t=0}$) and the end of the test ($C_{t=48}$).

Calculation of EC_{50}:

The EC_{50}-value was calculated at 48 hours of exposure from the probits of the percentages of affected daphnids and the logarithms of the corresponding WAFs prepared at the various loading rates using the maximum likelihood estimation method (Finney, D.J., 1971: Probit analysis, Cambridge University Press, Cambridge, U.K., 3rd edition).

6.8.2. Acceptability of the test

1. In the control, no daphnids became immobilised.
2. The oxygen concentration at the end of the test was ≥ 3 mg/l in control and test vessels.

6.9. List of deviations

6.9.1. List of protocol deviations

There were no deviations from the protocol.

6.9.2. List of standard operating procedures deviations

Any deviations from standard operating procedures were evaluated and filed in the study file. There were no deviations from standard operating procedures that affected the integrity of the study.

7. RESULTS

7.1. Combined limit/range-finding test

Table 1 shows the responses recorded during the combined limit/range-finding test. No significant immobility was observed after 24 hours in any of the test groups. Immobility in the WAFs prepared at 10 and 100 mg/l was complete after 48 hours, while 20% immobility was observed in the WAF prepared at 1.0 mg/l. Consequently, the EC_{50} was expected to be between concentrations present in WAFs prepared at 1.0 and 10 mg/l. Analyses of samples taken from the WAF prepared at 1.0 mg/l showed a measured concentration of 7.7 µg/l at the start that decreased to 1.1 µg/l after 48 hours of exposure (see also Table 2 of the appended Analytical report).

Immobility observed in the control group was within the limits prescribed for validity of the test. All test conditions were maintained within the limits prescribed by the protocol.

Table 1 Incidence of immobility in the combined limit/range-finding test

Loading rate [redacted] (oil free) WAF (mg/l)	Vessel number	Number Daphnia exposed	Response at 24 h		Response at 48 h	
			number	Total %	number	Total %
Control	A	5	0	5	1	10
	B	5	0		0	
	C	5	0		0	
	D	5	1		1	
1.0	A	5	0	20	0	20
	B	5	2		2	
10	A	5	0	0	5 (3)	100
	B	5	0		5 (2)	
100	A	5	0 (5)	0	5	100
	B	5	0 (5)		5	
	C	5	0 (5)		5	
	D	5	0 (5)		5	

( ) between brackets. number of daphnia observed trapped at the surface of the test solutions. These organisms were reimmersed into the respective solutions before recording of mobility.

7.2. Final test

7.2.1. Measured concentrations

The results of analysis of the samples taken during the final test are described in Table 3 of the appended Analytical Report.

Analyses were only considered necessary for the WAFs essential for determination of the toxicity parameters, i.e. the WAFs prepared at 4.6 and 10 mg/l. Analyses showed that the measured concentrations were 8.7 µg/l and 105 µg/l, respectively. The measured concentrations decreased to 1.8 and 17.5 µg/l during the test period in the WAFs of 4.6 and 10 µg/l, respectively. The observed decrease was likely related to the very low solubility (indicated as "insoluble" by the sponsor).The average exposure concentrations for the WAFs prepared at 4.6 and 10 mg/l were respectively 4.0 and 43 µg/l.

7.2.2. Immobility

Table 2 shows the responses recorded during the final test. The responses recorded in this test allowed for reliable determination of an EC_{50}. The responses recorded were in agreement with what was expected based on the results of the range-finding test.

Table 2 Acute immobilisation of daphnids after 24 and 48 hours in the final test

Loading rate [redacted] (oil free) WAF (mg/l)	Vessel number	Number Daphnia exposed	Response at 24 h		Response at 48 h	
			number	Total %	number	Total %
Control	A	5	0	0	0	0
	B	5	0		0	
	C	5	0 (1)		0 (2)	
	D	5	0		0 (1)	
0.46	A	5	0	0	0	0
	B	5	0		0	
	C	5	0		0	
	D	5	0		0	
1.0	A	5	2	15	2	15
	B	5	0 (1)		0	
	C	5	1		1	
	D	5	0		0	
2.2	A	5	0 (1)	0	0 (2)	0
	B	5	0		0	
	C	5	0		0	
	D	5	0		0	
4.6 [4.0 µg/l]	A	5	0 (1)	0	0	0
	B	5	0 (1)		0	
	C	5	0		0 (1)	
	D	5	0 (1)		0	
10 [43 µg/l)	A	5	0 (1)	0	5	85
	B	5	0 (3)		4 (3)	
	C	5	0 (1)		4	
	D	5	0 (3)		4 (3)	

( ) between brackets number of daphnia observed trapped at the surface of the test solutions These organisms were reimmersed into the respective solutions before recording of mobility.

[ ] Between brackets average exposure concentration

7.2.3. Determination of effect concentrations

Table 3 shows the effect parameters based on loading rates and average measured exposure concentrations, see also APPENDIX 1.

Table 3 Effect parameters

Parameter	Loading rate [redacted] (oil free) WAF (mg/l)	95%-confidence interval (mg/l)	Concentration [redacted] (oil free) (µg/l)	95%-confidence interval (µg/l)
NOEC	4.6	-	4.0	-
24h-EC_{50}	> 10	-	> 43	-
48h-EC_{50}	> 4.6, < 10	-	19	14-30

7.2.4. Experimental conditions

The results of measurement of pH and oxygen concentrations (mg/l) are presented in Table 4. These test conditions remained within the limits prescribed by the protocol (pH: 6.0-8.5, not varying by more than 1.5 unit; oxygen: ≥3 mg/l at the end of the test).

The temperature of the test medium was 20.3°C at the start of the test. The temperature continuously measured in a temperature control vessel varied between 19.8 and 20.4°C during the test, and complied with the requirements as laid down in the protocol (18-22°C, constant within 2°C).

Table 4 pH and oxygen concentrations during the final test

Loading rate [redacted] (oil free) WAF (mg/l)	Start (t=0 h)		End (t=48 h)	
	pH	O_2	pH	O_2
Control	7.8	10.6	8.0	9.1
0.46	7.8	10.1	8.1	9.2
1.0	7.8	10.0	8.1	9.2
2.2	7.9	9.9	8.1	9.1
4.6 [4.0 µg/l]	7.9	9.8	8.1	9.1
10 [43 µg/l]	7.9	9.8	8.2	9.2

[ ] Between brackets, average exposure concentration.

8. CONCLUSION

Under the conditions of the present study [redacted] (oil free) did not induce acute immobilisation of *Daphnia magna* exposed to a WAF prepared at a loading rate of 4.6 mg/l after 48 hours of exposure. Analyses showed that this corresponded to an average exposure concentration of 4.0 µg/l (NOEC).

The 48h-EC_{50} was between concentrations present in WAFs prepared at loading rates of 4.6 and 10 mg/l. The 48h-EC_{50} based on average exposure concentrations was 19 µg/l (95% confidence interval between 14 and 30 µg/l).

APPENDIX 1 EC-VALUES

Table 5 EC_{50} value at 48 hours and related parameters

```
48h-EC50 daphnia = 19.0 ug/L
95 % fiducial limits: 14.3 - 29.7 ug/L

index of regression significance: g=0.05
chi-squared=1.18, with 6 degrees of freedom

regression line: log10(conc.)=0.60+(probit-3.03)/2.92
```

conc. ug/L	group size	response	corrected fraction	expected fraction	chi2
4	5	0	0.00	0.00	0.00
4	5	0	0.00	0.00	0.00
4	5	0	0.00	0.00	0.00
4	5	0	0.00	0.00	0.00
43	5	5	1.00	0.85	0.88
43	5	4	0.80	0.85	0.10
43	5	4	0.80	0.85	0.10
43	5	4	0.80	0.85	0.10
					1.18

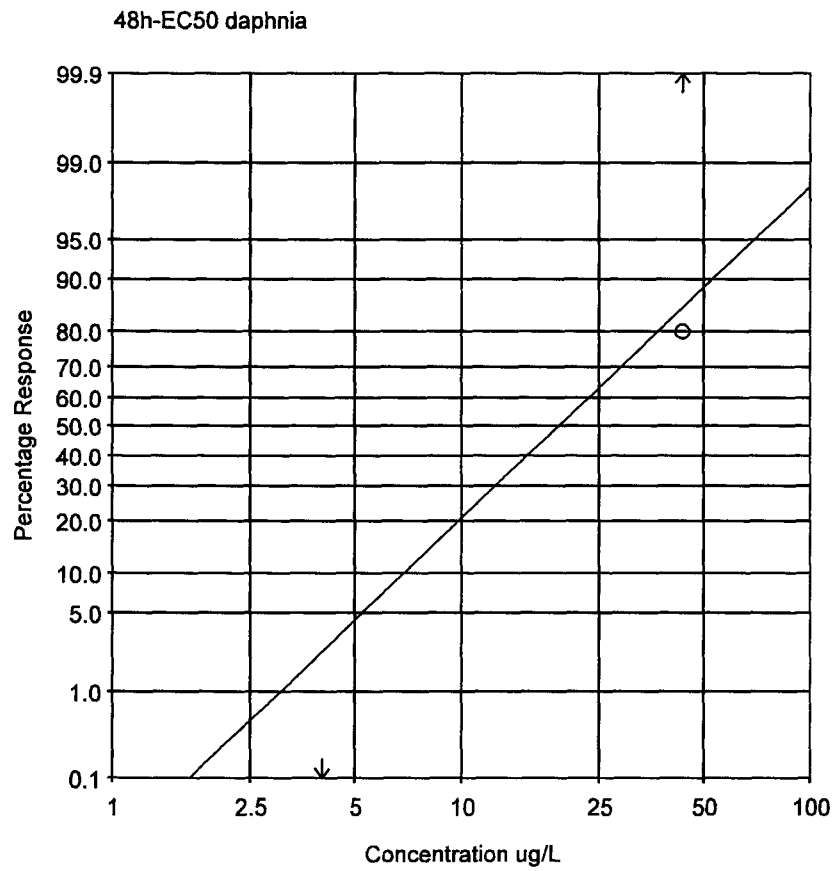


Figure 1 Percentage response (=immobility) of *Daphnia magna* as function of the log concentration of [redacted] (oil free) at 48h

APPENDIX 2 REFERENCE TEST

Start: 16 April 2012
End: 18 April 2012

48-hour Acute Toxicity Study in *Daphnia magna* with potassium dichromate ($K_2Cr_2O_7$) (NOTOX Project 499857).

The study procedures described in this report were based on the OECD guideline No. 202: "Daphnia sp., Acute Immobilisation Test", Adopted April 13, 2004 and the ISO International Standard 6341.

The reference test was carried out to check the sensitivity of the test system as used by NOTOX. Daphnia were exposed for a maximum of 48 hours to $K_2Cr_2O_7$ concentrations of 0.10, 0.18, 0.32, 0.56, 1.0 and 1.8 mg/l and to a blank control. Twenty daphnia were exposed per concentration.

The reference substance, potassium dichromate ($K_2Cr_2O_7$, art. 1.04864, batch no. K34869764 607) was obtained from Merck, Darmstadt, Germany.

Acute immobilization of daphnia after 24 and 48 hours in the reference test with potassium dichromate:

Concentration (mg/l)	Number Exposed	% immobile		Expected response (%) After 48 hours [1]	
		24h	48h	Minimal	Maximal
control	20	0	0	0	10[2]
0.10	20	0	0	0	10
0.18	20	0	0	0	10
0.32	20	0	85	0	30
0.56	20	75	95	0	100
1.0	20	100	100	40	100
1.8	20	100	100	100	100

[1] Based on historical data of the previous years (n>60)
[2] A maximum response of 10% does not invalidate the results of the test.

The actual responses in this reference test with $K_2Cr_2O_7$ were just outside (below) the ranges of the expected historical responses at the different concentrations, i.e. a 48h-EC_{50} between 0.3 and 1.0 mg/l. Hence, the sensitivity of this batch of *D. magna* was slightly higher when compared to the historical data collected at NOTOX.

The 24h-EC_{50} was 0.49 mg/l with a 95% confidence interval between 0.45 and 0.55 mg/l.

The 48h-EC_{50} was 0.28 mg/l with a 95% confidence interval between 0.14 and 0.65 mg/l.

The raw data from this study are kept in the NOTOX archives. The test described above was performed under GLP with a QA-check.

APPENDIX 3 ANALYTICAL REPORT

DETERMINATION OF THE CONCENTRATIONS

Author

E. Baltussen, PhD.

1. CONTENTS

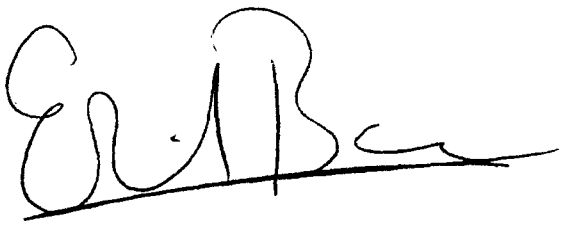
TABLES

2. REPORT APPROVAL

NOTOX B.V.

Principal Scientist
Analytical Chemistry

E. Baltussen, PhD.



Date: 20 August 2012

3. INTRODUCTION

3.1. Preface

Study plan analytical phase	Start	: 24 February 2012
	Completion	: 11 April 2012

3.2. Aim of the study

The purpose of the analytical phase was to determine the actual concentrations in samples taken from the test solutions used during the ecotoxicity test.

4. MATERIALS AND METHODS

4.1. Reagents

Water	Tap water purified by a Milli-Q water purification system (Millipore, Bedford, MA, USA).
Acetonitrile	Biosolve, Valkenswaard, The Netherlands.
Formic acid	Biosolve.
Tetrahydrofuran (THF)	VWR International, Leuven, Belgium.
ISO-medium	see main report.

All reagents were of analytical grade, unless specified otherwise.

4.2. Samples

The samples were stored in the freezer (≤ -15°C). Storage stability of samples under these conditions was demonstrated in NOTOX project 498463.

On the day of analysis, the samples were defrosted at room temperature. The test samples were diluted in a 1:3 (v:v) ratio with acetonitrile and analysed. If necessary, the samples were further diluted with 75/25 (v/v) acetonitrile/ISO-medium to obtain concentrations within the calibration range.

4.3. Analytical method

4.3.1. Analytical conditions

Quantitative analysis was based on the analytical method validated for the test substance in NOTOX project 498463.

Instrument	Acquity UPLC system (Waters, Milford, MA, USA)
Detector	Xevo TQ-S mass spectrometer (Waters)
Column	Acquity UPLC BEH C18, 100 mm × 2.1 mm i.d., dp = 1.7 µm (Waters)
Column temperature	40°C ± 1°C
Injection volume	5 µl
Mobile phase	0.05% formic acid in 85/15 (v/v) acetonitrile/water
Flow	0.5 ml/min
MS detection	
Ionisation source	ESI^+
Cone voltage	50 V
Collision energy	26
Quantitation	*m/z* 382.3 → *m/z* 200.1

4.3.2. Preparation of the calibration solutions

Stock and spiking solutions
Stock solutions of the test substance were prepared in THF at concentrations of 1020 – 1576 mg/l.

Spiking solutions were made up from a stock solution and/or dilutions of this solution. The solvent of the spiking solutions was THF.

Calibration solutions
Five solutions with the test substance in the concentration range of 0.02 - 3 mg/l were prepared in acetonitrile from two stock solutions. The solutions were 100-times diluted with 75/25 (v/v) acetonitrile/ISO-medium to obtain calibration solutions in the concentration range of 0.2 - 30 µg/l.

Procedural recovery samples
1 ml blank medium was spiked with the test substance at a target concentration of 0.01, 1 or 10 mg/l. The accuracy samples were treated similarly as the test samples (see paragraph 4.2 'Samples').

4.3.3. Sample injections

Calibration solutions were injected in duplicate. Test samples and procedural recovery samples were analysed by single injection.

4.4. Electronic data capture

System control, data acquisition and data processing were performed using the following programme:
- MassLynx version 4.1 (Waters, Milford, MA, USA)

Temperature, relative humidity and/or atmospheric pressure during sample storage and/or performance of the studies was monitored continuously using the following programme:
- REES Centron Environmental Monitoring system version SQL 2.0 (REES Scientific, Trenton, NJ, USA).

4.5. Formulas

Response (R) — Peak area test substance [units]

Calibration curve

$$R = a\,C_N + b$$

where:
C_N = nominal concentration [mg/l]
a = slope [units × l/mg]
b = intercept [units]

Analysed concentration (C_A)

$$C_A = \frac{(R - b)}{a} \times d \quad [mg/l]$$

where:
d = dilution factor

Recovery

$$\frac{C_A}{C_N} \times 100 \quad [\%]$$

Relative to nominal concentration

$$\frac{C_A}{C_N} \times 100 \quad [\%]$$

Relative to initial concentration

$$\frac{C_A\,(t = x\,\text{hours})}{C_A\,(t = 0\,\text{hours})} \times 100 \quad [\%]$$

5. RESULTS

5.1. Calibration curves

Calibration curves were constructed using five concentrations. For each concentration, two responses were used. Linear regression analysis was performed using the least squares method with a 1/concentration^2 weighting factor. The coefficient of correlation (r) was > 0.99 for each curve.

5.2. Samples

5.2.1. Procedural recovery samples

The results for the procedural recovery samples are given in Table 1.

The mean recoveries of the procedural recovery samples at 0.01 and 1 mg/l fell within the criterion of 70-110%. It demonstrated that the analytical method was adequate for the determination of the test substance in the test samples in this range.

The mean recovery of the procedural recovery samples prepared at 10 mg/l on 24-02-12 was 65%. The low solubility of the test substance in ISO-medium was probably the cause of this low recovery. However, the concentration in the test samples analysed on 24-02-12 was <0.01 mg/l and since the recovery of the procedural recovery samples at 0.01 mg/l was between 70-110%, the analytical method was considered to be adequate for the determination of the test substance in these test samples.

5.2.2. Test samples

The results for the test samples are given in Table 2 and Table 3.

6. TABLES

Table 1 Procedural recovery samples

Date of preparation [dd-mm-yy]	Date of analysis [dd-mm-yy]	Target concentration [mg/l]	Nominal concentration [mg/l]	Analysed concentration [mg/l]	Recovery [%]	Mean recovery [%]
24-02-12	24-02-12	0.01	0.0102 0.0102	0.0106 0.0102	104 100	102
24-02-12	24-02-12	10	10.2 10.2	6.39 6.93	63 68	65
11-04-12	11-04-12	0.01	0.00997 0.00997	0.0101 0.0103	101 103	102
11-04-12	11-04-12	1	1.00 1.00	0.999 1.02	100 102	101

Table 2 Concentrations of the test substance in test medium - combined limit/range-finding test

Time of sampling [hours]	Date of sampling [dd-mm-yy]	Date of analysis [1] [dd-mm-yy]	Loading rate [2] [mg/l]	Concentration analysed [mg/l]	Relative to initial [%]
0	13-02-12	24-02-12	1	0.00767	
48	15-02-12	24-02-12	1	0.00109	14

[1] Samples were stored in the freezer (≤ -15°C) until the day of analysis.

[2] A water accommodated fraction (WAF) prepared at the loading rate.

Table 3 Concentrations of the test substance in test medium - final test

Time of sampling [hours]	Date of sampling [dd-mm-yy]	Date of analysis [1] [dd-mm-yy]	Loading rate [2] [mg/l]	Concentration analysed [mg/l]	Relative to initial [%]
0	12-03-12	11-04-12	0 4.6 10	0.000572[3] 0.00876 0.105	
48	14-03-12	11-04-12	0 4.6 10	n.d. 0.00179 0.0175	n.a. 20 17

[1] Samples were stored in the freezer (≤ -15°C) until the day of analysis.

[2] A water accommodated fraction (WAF) prepared at the loading rate.

[3] Obtained by extrapolation of the calibration curve.

n.d. Not detected.

n.a. Not applicable.

APPENDIX 4 PROTOCOL

PROTOCOL

Study Title

ACUTE TOXICITY STUDY IN *DAPHNIA MAGNA* WITH [redacted] (OIL FREE) (STATIC)

Author

Ing. M.H.J. Migchielsen

Test Facility

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Hambakenwetering 7
5231 DD 's-Hertogenbosch
The Netherlands

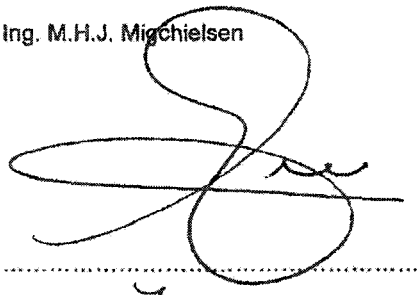
Laboratory Project Identification

NOTOX Project 498470
NOTOX Substance 203662/A

1. CONTENTS

2. PROTOCOL APPROVAL

STUDY DIRECTOR: Ing. M.H.J. Migchielsen



date: 19 January 2012

HEAD OF QUALITY ASSURANCE: C.J. Mitchell B.Sc.

date: 20-Jan-2012

SPONSOR: [redacted]

date: 30-Jan-2012

3. INTRODUCTION

3.1. Preface

Sponsor	[redacted]
Study Monitor	Mr. R. Balcomb Director, Toxicology and Environmental Assessments Intertek Regulatory Services 1035 17th Street No.4 SANTA MONICA, CA 90403 USA
Test Facility	NOTOX B.V. Hambakenwetering 7 5231 DD 's-Hertogenbosch The Netherlands
Study Director	Ing. M.H.J. Migchielsen Marcel.migchielsen@notox.nl
Technical Coordinator	Ing. V.E. Carolus
Principal Scientist	Dr. K.A. Oudhoff
Study Plan	Start week beginning : 20 February 2012 (week 08) Completed week beginning : 26 March 2012 (week 13) Proposed Reporting date : 06 May 2012

3.2. Aim of the study

The purpose of the study is to evaluate the test substance for its ability to generate acute toxic effects on the mobility of *Daphnia magna* during an exposure period of 48 hours and, if possible, to determine the EC_{50} at 24 and 48 hours of exposure.

3.3. Guidelines

The study procedures described in this protocol are based on the Organization for Economic Co-operation and Development (OECD), OECD guidelines for Testing of Chemicals, guideline No. 202: "*Daphnia sp.*, Acute Immobilisation Test", Adopted April 13, 2004.

In addition, the procedures are designed to meet the test methods and validity criteria prescribed by the following guidelines:

- ISO International Standard 6341: "Water quality - Determination of the inhibition of the mobility of *Daphnia magna* Straus (Cladocera, Crustacea) - Acute toxicity test, Third edition, 1996-04-01.
- Commission Regulation (EC) No 440/2008 of 30 May 2008, Part C: Methods for the determination of ecotoxicity, Publication No. L142, C.2. "Daphnia Sp. Acute Immobilisation Test".

And, if applicable, the following guidance document will be followed:

- Guidance document on aquatic toxicity testing of difficult substances and mixtures, OECD series on testing and assessment number 23, December 14, 2000.

3.4. Good Laboratory Practice

The study will be performed according to:

The Organization for Economic Cooperation and Development (OECD) Good Laboratory Practice Guidelines (1997).

Which essentially conform to:

The United States Food and Drug Administration Good Laboratory Practice Regulations.

The United States Environmental Protection Agency Good Laboratory Practice Regulations.

3.5. Quality Assurance

Study and/or process inspections will be performed by the NOTOX Quality Assurance Unit to assure the GLP compliance of this study. Facility inspections are also performed at regular intervals to assure the GLP compliance of general aspects.

The protocol will be inspected to confirm that it complies with GLP regulations. The report will be inspected to confirm that the methods and results accurately and completely reflect the raw data.

3.6. Storage and retention of records and materials

Records and materials pertaining to the study, including protocol, raw data, specimens (except specimens requiring refrigeration or freezing) and the final report, will be retained in the NOTOX archives for a period of at least 2 years after finalization of the report. After this period, the sponsor will be contacted to determine how the records and materials should be handled. NOTOX will retain information concerning decisions made.

Those specimens requiring refrigeration or freezing will be retained by NOTOX for as long as the quality of the specimens permits evaluation but no longer than three months after finalization of the report.

NOTOX will retain a test substance sample until the expiry date, but no longer than 10 years after finalization of the report. After this period the sample will be destroyed.

3.7. Definitions

Immobile are those animals not able to swim within 15 seconds after gentle agitation of the test vessel.

The **EC_{50}** is the concentration of test substance estimated to immobilise 50% of the daphnia after a defined period of exposure.

No Observed Effect Concentration (NOEC) is the highest concentration tested at which no effect (i.e. immobilisation) is recorded.

If appropriate, additional definitions may be included in the report (e.g. definitions referring to poorly soluble substances).

4. MATERIALS AND METHODS

4.1. Test substance

The sponsor is responsible for Good Laboratory Practice (GLP) compliance for all test substance information unless determined by NOTOX. This will be specified in the GLP compliance statement in the report.

4.1.1. Test substance information

Identification	[redacted] (oil free)
Molecular formula	[redacted]
CAS Number	1159919-46-6
Description	Clear yellow viscous liquid (determined at NOTOX)
Batch	PB-39-131
Purity	UVCB
Test substance storage	At room temperature in the dark
Stability under storage conditions	Stable
Expiry date	01 December 2012 (allocated by NOTOX, 1 year after receipt of the test substance)

4.1.2. Study specific test substance information

Hygroscopic	Not indicated
Volatile	Not indicated
Density	1.23 g/mL
Stability at higher temperatures	Not indicated
Stability in vehicle:	
• Water	Not indicated
• Dimethyl sulphoxide	Not indicated
• Ethanol	Not indicated
• Acetone	Not indicated
Solubility in vehicle:	
• Water	Insoluble
• Dimethyl sulphoxide	Soluble when hot
• Ethanol	Soluble
• Acetone	Soluble

Cite publicly per PMN and TSCA Inventory list nomenclature as: Amines, bis (C11-14-branched and linear alkyl) tungstates.

4.1.3. Safety precautions and disposal category

Safety precautions	Gloves, goggles and face mask to ensure personnel health and safety
Disposal category	III

4.1.4. Reference substance

Results of a recently performed reference test with potassium dichromate will be included in the report. Reference tests will be performed once every three months.

4.2. Test system

Species	*Daphnia magna* (Crustacea, Cladocera) (Straus, 1820), at least third generation, obtained by acyclical parthenogenesis under specified breeding conditions.
Source	In-house laboratory culture with a known history.

Reason for selection	This system has been selected as an internationally accepted invertebrate species.
Validity of batch	Daphnids will originate from a healthy stock, 2nd to 5th brood, showing no signs of stress such as mortality >20%, presence of males, ephippia or discoloured animals and there should be no delay in the production of the first brood.
Characteristics	For the test selection of young daphnia with an age of < 24 hours, from parental daphnids of more than two weeks old.

4.3. Breeding

Start of each batch	With newborn daphnids, i.e. less than 3 days old, by placing about 250 of them into 5 litres of medium in an all-glass culture vessel.
Maximum age of the cultures	4 weeks
Renewal of the cultures	After 7 days of cultivation half of the medium twice a week.
Temperature of medium	18-22°C
Feeding	Daily, a suspension of fresh water algae.
Medium	M7, as prescribed by Dr. Elendt-Schneider (Elendt, B.-P., 1990: Selenium deficiency in Crustacea. An ultrastructural approach to antennal damage in *Daphnia magna* Straus. Protoplasma 154, 25-33).

Composition of medium M7

Adjusted ISO medium. the following chemicals (analytical grade) are dissolved in tap water purified by Reverse Osmosis (RO-water, GEON Waterbehandeling, Berkel-Enschot, The Netherlands).

Macro salts	$CaCl_2\ 2H_2O$	211 5	mg/l
	$MgSO_4\ 7H_2O$	88 8	mg/l
	$NaHCO_3$	46 7	mg/l
	KCl	4 2	mg/l

Medium M7 trace elements, macro nutrients and vitamins are added to freshly prepared ISO medium to reach the following concentrations.

Trace elements	B	0.125	mg/l
	Fe	0 05	mg/l
	Mn	0 025	mg/l
	Li, Rb and Sr	0.0125	mg/l
	Mo	0 0063	mg/l
	Br	0 0025	mg/l
	Cu	0 0016	mg/l
	Zn	0 0063	mg/l
	Co and I	0 0025	mg/l
	Se	0 0010	mg/l
	V	0 0003	mg/l
	$Na_2EDTA.2H_2O$	2 5	mg/l
Macro nutrients	$Na_2SiO_3\ 9H_2O$	10 0	mg/l
	$NaNO_3$	0 27	mg/l
	KH_2PO_4	0 14	mg/l
	K_2HPO_4	0 18	mg/l

Vitamins	Thiamine	75 0	µg/l
	B_{12}	1 0	µg/l
	Biotin	0 75	µg/l

The hardness approximately 180 mg/l expressed as $CaCO_3$ and the pH 7 7 ± 0 3

4.4. Preparation of stock and test solutions

The procedure for preparation of test solutions will be based on the available test substance information and/or on a pre-test.

The standard test procedures require generation of test solutions, which contain completely dissolved test substance concentrations or stable, and homogeneous mixtures or dispersions. The testing of concentrations that disturb the test system will be prevented or avoided, e.g. film of the test substance on the water surface or extensive precipitation, flocculation, aggregation or deposition of the undissolved fraction of the test substance. The method of preparation of the test solutions will be based on data of the test substance supplied by the sponsor and/or on the results of a preliminary test (or specific tests with the test substance performed by NOTOX when the sponsor requests these).

If applicable, the method of preparation will alternatively be based on the principles laid down in the OECD Guidance Document on Aquatic Toxicity Testing of Difficult Substances and Mixtures and referred to in the OECD Guidance Document On The Use Of The Harmonised System For The Classification Of Chemicals Which Are Hazardous For The Aquatic Environment, section 3.5: "Difficult to test substances".

The tests will be carried out without adjustment of the pH, except if pH values of test solutions are outside the optimal pH range for the species to be tested.

4.5. Range-finding test

A range-finding test will be performed to provide information about the range of concentrations to be used in the final test. Test procedure and conditions will be similar to those applied in the final test with the following exceptions:
Ten daphnia per concentration (5 per vessel) will be exposed to a range of 0.1 to 100 mg/l increasing by a factor of 10. If applicable a range will be tested up to and including the maximum solubility if this is below 100 mg/l. Dissolved oxygen concentrations and pH will at least be measured in the control and the highest test concentration.

During the exposure period the actual test concentrations should be maintained at 80% or more of the initial concentrations. Determination of the stability of the test substance under test conditions will be performed using samples taken at t= 0 and 48 hours during the range-finding test. The decision which samples will be taken for analysis is made at the start of the test in case samples need to be analysed freshly. Standard procedures assume stability of the test substance under deep freeze conditions. If stability is guaranteed, samples will be taken from all concentrations (except control(s)). The decision which samples will be analysed is then taken by the Study Director at the end of the test period based on the biological results. Optionally, sampling at the end of the test period may be limited to the concentration(s) that are chosen for analysis.
If applicable, the frequency of renewal of the test solutions to be used in the final study will be defined according to the results of this analysis (See also additional procedures).

Depending on the solubility of the test substance in test medium and, if appropriate the expected level of toxicity, the range of concentrations in the range-finding test may be different or include less concentrations. Alternatively, different methods of preparation may be combined in order to reach the expected water solubility (and dilutions thereof).

If no toxicity is expected, based on the characteristics of the test substance or other specific information, a limit test or alternatively a limit test combined with a range-finding test will be performed.

4.5.1. Limit test

The limit test will consist of a blank-control and a concentration of 100 mg/l or, if applicable, a saturated solution whichever is lower. Test procedure and conditions will be similar to those applied in the final test. Samples for determination of actual exposure concentrations will be taken from the control and the test concentration at the start and the end of the test. No further testing will be required if no effects are observed and the validity criteria are met.

Depending on the solubility of the test substance in test medium different methods of preparation may be combined in order to reach the expected water solubility.

4.5.2. Combined limit/range-finding test

In a combined limit/range-finding test, twenty Daphnia per concentration will be exposed to a blank-control and a concentration of 100 mg/l or, if applicable, a saturated solution whichever is lower. Ten daphnia per concentration will be exposed to 0.1, 1.0 and 10 mg/l, or if applicable dilutions containing 0.1, 1.0 and 10% of the saturated solution. Dissolved oxygen concentrations and pH will at least be measured in the control and the highest test concentration. Samples for determination of actual exposure concentrations will be taken from at least the control and the highest test concentration at the start and the end of the test. No further testing will be required if no effects are observed and the validity criteria are met.

Depending on the solubility of the test substance in test medium and, if appropriate the expected level of toxicity, the range of concentrations in the range-finding test may be different or include less concentrations. Alternatively, different methods of preparation may be combined in order to reach the expected water solubility (and dilutions thereof).

4.6. Final test

4.6.1. Test concentrations

Number	At least 5 concentrations in a geometric series with a factor ≤ 2.2, except when the EC_{50} is expected to be greater than the maximum concentration to be tested. In that case a limit test can be performed.
Range	Preferably, the concentration range has to cover at least one concentration causing no immobility, one or more concentrations causing 10 to 90% immobility and one concentration causing 100% immobility with a standard maximum concentration of 100 mg/l or, if applicable, a saturated solution whichever is lower. The range may however include concentrations above 100 mg/l if this is relevant for the calculation of an EC_{50}-value.
Controls	Test medium without test substance or other additives or, if relevant, a control containing test medium with the additive used in the treatment of the stock solutions.

4.6.2. Test procedure and conditions

Test duration	48 hours
Test type	Static
Test vessels	100 ml, all-glass
Medium	Adjusted ISO medium

Number of daphnia	20 per concentration
Loading	5 per vessel (with ca. 80 ml of test solution)
Light	16 hours photoperiod daily; should the test substance be light sensitive, the test will be performed in the dark.
Temperature	18-22°C, constant within 2°C
Oxygen concentration	≥ 3 mg/l at the end of the test.
pH	Between 6.0 and 8.5. Should not vary by more than 1.5 unit at the end of the test in any test solution.
Feeding	No feeding
Aeration	No aeration of the test solutions.
Introduction of daphnia	Daphnia are introduced into the test medium as soon as possible after preparation of the test solutions.

4.6.3. Sampling for analysis of test concentrations

Frequency	At the start (t=0 h) and the end of the test (t=48 h). If analytical results show that a concentration has decreased below the LOD/LOQ before the end of the test period, no further sampling is needed at that concentration.
Concentrations (standard[1])	Samples for analysis will be taken from three concentrations, i.e. the lowest, a middle and the highest, and the control. At the start of the test care will be taken not to include any floating layer, test substance film or undissolved material in separate vessels. At the end of the test samples will be taken from the approximate centre of the pooled solutions (provided that solutions are still homogeneous) of the vessels at each concentration.
Number of samples	Sampling will consist of singular samples per treatment. Should the analytical validation require duplicate or multiple samples per treatment, this will be followed without prior notification. In case undissolved particles were removed from the test solutions before the start of the test, this residue will be retained for possible analysis.
Volume	Standardly, volumes of 2 ml will be taken, but depending on the limit of detection of the analytical method used in relation to the test concentrations the volume may differ.

[1] The standard frequency of sampling is only applicable provided that test solutions are diluted using **one** stock and test concentrations should be **above** 1 mg/l Sampling will include **all** test solutions if the previous mentioned conditions are not met or if the Study Director decides this is essential for other reasons. Alternatively, sampling may be limited to those test solutions that are biologically relevant.

Storage	If stability of test concentrations under deep-freeze conditions is ensured, the samples will be stored in a deep-freezer until analysis. Optionally, samples can be stored under different conditions (e.g. room temp. or in refrigerator) if stability under these conditions is ensured.
Extra samples	In case singular samples are taken, which are known to be stable under the storage conditions, extra samples will be taken from all concentrations and stored for possible analysis until delivery of the final report with a maximum of three months.
Analyses	Preferably, the entire volume of each sample used for analysis will be taken for further dilution or pre-treatment. The analytical method used will be based on the results of a separate project for the development and validation of the analytical method. If study specific adjustments of the analytical method or sample pre-treatment procedures are necessary, these will be developed and tested before the performance of the final test. Detailed specification of these additional analytical procedures will be put down in a protocol amendment (see also 'Additional procedures').

4.6.4. Measurements and recordings

Immobility (including mortality)	At 24 hours and at 48 hours.
pH	At the beginning and at the end of the test, for all concentrations and the control.
Dissolved oxygen	At the beginning and at the end of the test, for all concentrations and the control. In addition after 24 hours, immediately after counting the immobilized daphnids oxygen levels will be measured in the test container with the solution of lowest concentration at which all the *Daphnia magna* have been immobilized.
Temperature of medium	Continuously in a temperature control vessel, beginning at the start of the test.

4.7. Specific items for Study Director approval in study files

The following items will be approved in the study files by the Study Director:

- Choice of range-finding test, combined limit/range-finding test or limit test
- Concentrations to be tested
- Procedure(s) for preparation of test solutions
- Sampling and analysis:
  - Number and volume of samples to be taken
  - Treatment of samples
  - Samples to be analysed

4.8. Electronic data capture

Observations/measurements in the study will be recorded electronically using the following programme(s):
REES Centron Environmental Monitoring system version SQL 2.0 (REES Scientific, Trenton, NJ, USA): Temperature.

For the analytical instruments a selection of the following programmes will be used. The actual programme(s) will be approved in the raw data and reported.

- Empower version 7.00 (Waters, Milford, MA, USA)
- Enhanced Chemstation version D.00.01.27 (Agilent Technologies, Wilmington, DE, USA)
- MassLynx version 4.1 (Waters, Milford, MA, USA)
- Xcalibur version 2.0 (Thermo, San Jose, CA, USA)
- ICP-MS Chemstation version B.03.04 (Agilent Technologies, Tokyo, Japan)
- ICP-MS Chromatographic software version C.01.00 (Agilent Technologies, Tokyo, Japan)

Any upgrades will be approved by the Study Director (or Principal Scientist/Investigator) in the study files.

4.9. Interpretation

4.9.1. Acceptability of the test

1. In the control, and if applicable the solvent-control, not more than 10% of the daphnia should have been immobilised.
2. The dissolved oxygen concentration at the end of the test will be ≥ 3 mg/l in control and test vessels.

If (one of) the acceptability criteria are not met and the Study Director decides that this has a critical effect on the study, the test will be rejected and repeated.

4.9.2. Additional procedures

Additional or alternative procedures will be required for the testing of:
1. Volatile substances;
2. Very toxic or low soluble substances (test concentrations < 1 mg/l);
3. Hydrolytically unstable substances;
4. pH affecting substances;
5. Substances that are not stable under deep-freeze conditions.

These additional procedures may require the amending of:
1. Preparation of test solutions;
2. Additional testing for determination of stability of exposure concentrations;
3. Procedures for maintenance of exposure concentrations (semi-static or flow-through system);
4. The frequency of sampling and analysis for the determination of actual test concentrations;
5. Extension of the analytical program with respect to sample treatment and the sensitivity of the analytical method;
6. If applicable, additional testing with pH adjustment.

The additional procedures are no part of the standard test procedures and will be applied only after emission of an authorised protocol amendment. In such a case the amended procedures will be effective only after NOTOX has received any kind of authorisation from the study monitor.

4.9.3. Data handling

Defining exposure concentrations

1. The results will be based on the nominal or initial (if not in agreement with nominal) test substance concentrations if the analytical program has confirmed that the measured test substance concentrations remained within 20% of the nominal or initial concentrations.
2. If the deviation of the exposure concentrations of the test substance is greater than ± 20% of the nominal or initial concentrations, the results will be expressed in terms of average exposure concentrations. Where measured data are available for the start and end of the test, these concentrations are geometric means calculated from the concentrations measured at the start and end of the test. Where at the end of the test measured concentrations are below the analytical detection limit, such concentrations shall be considered to be half that detection limit.

Calculation of EC_{50}

If possible, an EC_{50}-value will be calculated at 24 and 48 hours of exposure from the probits of the percentages of affected daphnia and the logarithms of the corresponding nominal concentrations using the maximum likelihood estimation method (Finney, D.J., 1971: Probit analysis, Cambridge University Press, Cambridge, U.K., 3rd edition) or using the Logit-model (Cox, D.R., 1977: Analysis of binary data, Methuen & Co. Ltd.).

Should there be no concentration between the highest concentration (A) at which 0% immobility has occurred and the lowest concentration (B) at which 100% immobility has occurred, the EC_{50} will be calculated as $(AB)^{½}$, where A and B are limits of the 95% confidence interval.

No EC_{50} can be calculated if the test substance proves to be non-toxic (EC_{50} > maximum concentration).

Optionally, other statistical analyses may be performed if appropriate.

5. DISTRIBUTION

Original:	Study Director
1 Copy:	Technical Coordinator
1 Copy :	Analytical Chemistry
1 Copy:	QAU/Management
1 Copy:	Sponsor

PROTOCOL AMENDMENT NO : 1

Study Title	ACUTE TOXICITY STUDY IN *DAPHNIA MAGNA* WITH VANLUBE®W-324 (oil free) (STATIC)
Sponsor	[redacted]
Study Monitor	Mr. R. Balcomb Director, Toxicology and Environmental Assessments Intertek Regulatory Services 1035 17th Street No.4 SANTA MONICA, CA 90403 USA
NOTOX Substance	203662/A
NOTOX Project	498470

AMENDMENT DESCRIPTION

1. Page 4, Preface, Principal Scientist:

   Principal scientist will be changed to E. Baltussen, PhD.

REASONS FOR AMENDMENT

1. Formal replacement as K.A. Oudhoff, PhD is no longer working for NOTOX B.V.

APPROVAL
Study director

M.H.J. Mgchielsen, Bachelor

17 August 2012
date: